

Customer Number: 000959

**DIVISIONAL-CONTINUATION APPLICATION TRANSMITTAL FORM**  
**UNDER RULE 1.53(b) (former Rule 1.60)**

A

06/18/99  
 Jc520 U.S. PTO

DOCKET NUMBER	ANTICIPATED CLASSIFICATION OF THIS APPLICATION:		PRIOR APPLICATION SERIAL NUMBER: 08/312,429	PRIOR APPLICATION FILING DATE: SEPTEMBER 26, 1994
IGI-001CN3	CLASS:	SUBCLASS:	EXAMINER: S. PRIEBE	ART UNIT: 1632

ASSISTANT COMMISSIONER FOR PATENTS  
 BOX PATENT APPLICATION  
 WASHINGTON, DC 20231

**CERTIFICATION UNDER 37 CFR 1.10**

Date of Deposit: June 18, 1999Mailing Label Number: EL266738477US

I hereby certify that this 37 CFR 1.53(b) request and the documents referred to as attached therein are being deposited with the United States Postal Service on the date indicated above in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR 1.10 and addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Deise K. Timas  
 Name of Person Mailing Paper

Deise K. Timas  
 Signature of Person Mailing Paper

Jc503 U.S. PTO  
 09/335956  
 06/18/99

Dear Sir:

This is a request for filing a ☒ continuation ☐ divisional application under 37 CFR 1.53(b), of pending prior application serial no. 08/312,429 filed on September 26, 1994, of David Ward et al. entitled Delineation of Individual Human Chromosomes in Metaphase and Interphase Cells by In-Situ Suppression Hybridization which in turn is a continuation application of serial no. 07/837,664 filed on February 14, 1992, which in turn is a continuation-in-part of 07/271,609 filed November 15, 1988, now abandoned.

1. ☒ Enclosed is a copy of the latest inventor signed application, including the oath or declaration as originally filed. The copy of the enclosed papers is as follows:

- ☒ 79 page(s) of specification
- ☒ 5 page(s) of claims
- ☒ 1 page(s) of abstract
- ☒ 15 sheet(s) of drawing
- ☒ 18 page(s) of executed declaration and power of attorney
- ☒ 27 page(s) of Preliminary Amendment filed February 14, 1992.

I hereby verify that the attached papers are a true copy of the prior complete application serial no. 07/271,609 as originally filed on November 15, 1988 and a copy of the preliminary amendment filed in serial no. 07/837,664 as originally filed on February 14, 1992.

2. ☐ A verified statement to establish small entity status under 37 CFR 1.9 and 1.27, a copy of which is enclosed, was filed in the prior application and such status is still proper and desired (37 CFR 1.28(a)).
3. ☒ The filing fee is calculated below:

	NUMBER OF CLAIMS FILED			NUMBER EXTRA
TOTAL	* 18	MINUS	** 20	= 0
INDEP	* 8	MINUS	*** 3	= 5
<input type="checkbox"/> MULTIPLE DEPENDENT CLAIMS				

## SMALL ENTITY

RATE	FEE
x 9 =	\$0.00
x 39 =	\$0.00
+130 =	\$0.00
BASIC FEE	\$0.00
TOTAL	\$0.00

OR

## OTHER THAN A SMALL ENTITY

RATE	FEE
x 18 =	\$0.00
x 78 =	\$390.00
+ 260 =	\$0.00
BASIC FEE	\$760.00
TOTAL	\$1150.00

OR

4. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 12-0080. A duplicate copy of this sheet is enclosed.
5. ☒ A check in the amount of \$1150.00 is enclosed for payment of the filing fee.
6. ☐ Cancel in this application original claims \_\_\_\_\_ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)
7. ☐ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claims in the prior application.)
8. ☒ Amend the specification by inserting before the first line the sentences: "This application is a continuation application of serial no. 08/312,429 filed on September 26, 1994, Allowed, which in turn is a continuation application of serial no. 07/837,664 filed on February 14, 1992, which in turn is a continuation-in-part of 07/271,609 filed November 15, 1988, now abandoned. The contents of all of the aforementioned application(s) are hereby incorporated by reference."
9. ☐ Please abandon said prior application as of the filing date accorded this application. A duplicate copy of this transmittal is enclosed for filing in the prior application file. (May be used if signed by person authorized by §1.138 and before payment of base issue fee.)
10. ☐
11. ☐ Priority of application serial no. \_\_\_\_\_ filed on \_\_\_\_\_ in \_\_\_\_\_ is claimed under 35 U.S.C. §119.
  - ☐ The certified copy has been filed in prior application serial no. \_\_\_\_\_ filed on \_\_\_\_\_.
  - ☐ The certified copy will follow.
12. ☒ The prior application is assigned of record to Yale University.
13. ☒ Also enclosed is/are a substitute specification which incorporates the amendments to the original attached specification which were filed on February 14, 1992, January 23, 1995, and April 29, 1996.
14. ☒ Also enclosed is/are a copy of the amendments to the original specification which were filed on February 14, 1992, January 23, 1995 and April 29, 1996 and which have been incorporated into the substitute specification.
15. ☒ The power of attorney in the prior application is to Lahive & Cockfield, LLP.
  - a. ☒ The power appears in the original papers in the prior application.
  - b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.
  - c. ☐ A new power has been executed and is attached.
16. ☒ Also enclosed is an associate power of attorney filed in the prior application.
17. ☒ Also enclosed is a copy of the Assignment from the parent application (08/312, 429) which was recorded 2/18/99 at Reel 9778, Frame 0675 (23 pages).
18. ☒ Address all future communications (May only be completed by applicant, or attorney or agent of record) to Giulio A. DeConti, Jr. at **Customer Number: 000959** whose address is:

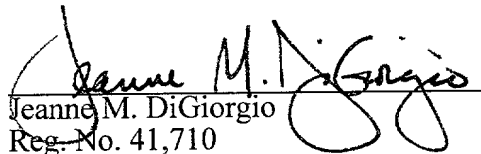
Lahive & Cockfield, LLP  
 28 State Street  
 Boston, Massachusetts 02109

19. ☒ Any requests for extensions of time necessary in a parent application for establishing copendency between this application and a parent application are hereby requested and the Commissioner is authorized to charge any fee associated with such an extension to Deposit Account No. 12-0080.
20. ☐ Pursuant to 37 CFR 1.821(e), the computer readable form of the sequence listing for this new application is to be identical with the computer readable form of application serial no. \_\_\_\_\_. Please use the computer readable form of application serial no. \_\_\_\_\_ in lieu of filing a duplicate computer readable form in this application. Pursuant to 37 CFR 1.821(f), the content of the paper copy of the sequence listing for this new application and the computer readable form of application serial no. \_\_\_\_\_ are the same.

Date

18 June 1999

LAHIVE & COCKFIELD, LLP  
28 State Street  
Boston, Massachusetts 02109  
Tel. (617) 227-7400

  
Jeanne M. DiGiorgio  
Reg. No. 41,710

- ☐ inventor(s) ☐ filed under §1.34(a)  
☐ assignee of complete interest  
☒ attorney or agent of record

## LAHIVE & COCKFIELD

JOHN A. LAHIVE, JR.  
W. HUGO LIEPMANN  
JAMES E. COCKFIELD  
THOMAS V. SMURZYNSKI  
RALPH A. LOREN  
THOMAS J. ENGELLENER  
WILLIAM C. GEARY III  
DAVID J. POWSNER  
GIULIO A. DeCONTI, JR.  
MICHAEL I. FALKOFF  
ANN LAMPORT HAMMITTE  
JOHN V. BIANCO  
AMY BAKER MANDRAGOURAS  
ELIZABETH A. HANLEY  
ANTHONY A. LAURENTANO  
PAUL LOUIS MYERS, Ph. D.

COUNSELLORS AT LAW  
SIXTY STATE STREET  
BOSTON, MASSACHUSETTS 02109-1875

TELEPHONE (617) 227-7400  
TELEX 752806  
FAX (617) 227-5941

January 23, 1995

JANE E. REMILLARD  
EDWARD J. KELLY  
MARK A. KURISKO  
BETH E. ARNOLD  
JEAN M. SILVERI

OF COUNSEL  
JEREMIAH LYNCH

PATENT AGENT  
MATTHEW P. VINCENT, Ph. D.

TECHNICAL SPECIALISTS  
CATHERINE J. KARA, Ph. D.  
MARK D. RUSSETT  
CAROLYN G. d'AGINCOURT  
ISABELLE M. CLAUS, Ph. D.  
IVANA M. MAGOVCEVIC, Ph. D.

### VIA FACSIMILE

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

COPY

Re: U.S. Patent Application Serial No. 08/312,429  
*Delineation of Individual Human Chromosomes in Metaphase  
and Interphase Cells by In Situ Suppression Hybridization*  
Filed on: September 26, 1994  
By: David C. Ward et al.  
Our Ref.: IGI-001CN2

Dear Sir:

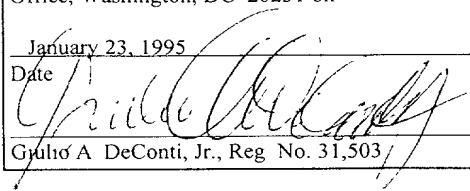
I enclose herewith for filing in the above-identified application the following:

1. Preliminary Amendment and Remarks

Please charge any necessary fees to our Deposit Account No. 12-0080. A duplicate of this sheet is enclosed.

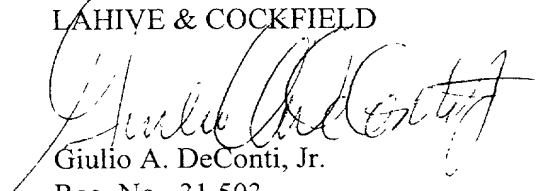
#### CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being facsimile transmitted to the Commissioner of Patents and Trademarks Office, Washington, DC 20231 on:

January 23, 1995  
Date  
  
Giulio A. DeConti, Jr., Reg. No. 31,503

Respectfully submitted,

LAHIVE & COCKFIELD

  
Giulio A. DeConti, Jr.  
Reg. No. 31,503



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: David C. Ward, Peter Lichter, Thomas Cremer, Laura Manuelidis,  
Thomas Ried and Antonio Baldini

Serial No.: 08/312,429

Group Art Unit: 1807

Filed: September 26, 1994

Examiner: M. Fleisher

Attorney's Docket No.: IGI-001CN2

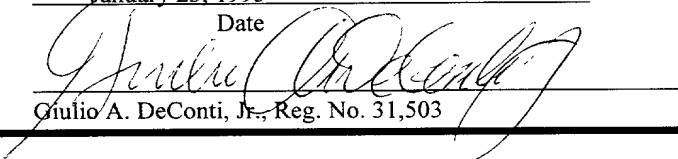
Title: *Delineation of Individual Human Chromosomes in Metaphase  
and Interphase Cells by In Situ Suppression Hybridization*

**CERTIFICATE OF FACSIMILE TRANSMISSION**

I hereby certify that this correspondence is being facsimile transmitted to the Honorable Commissioner of Patents and Trademarks, Washington, D.C. 20231 on the date set forth below.

January 23, 1995

Date

  
Giulio A. DeConti, Jr., Reg. No. 31,503

**PRELIMINARY AMENDMENT PURSUANT TO 37 C.F.R. § 1.115**

The Honorable Commissioner  
of Patents and Trademarks  
Washington, D.C. 20231

Dear Sir:

Prior to examination, please amend patent application Serial No.  
08/312,429, filed September 26, 1994, which is a file wrapper continuation of  
patent application Serial No. 07/837,664, filed February 14, 1992, now

abandoned, which is a file wrapper continuation of patent application Serial No. 07/271,609 filed November 15, 1988, now abandoned, as follows:

In the Specification

On page 4, under the heading Brief Description of the Drawings, please delete page 4, line 5 through page 12, line 12 and insert:

---Figure 1 presents an outline of CISS hybridization for specific staining of human chromosomes.

Figures 2A-2F show suppression from cross-reacting sequences within a chromosome 7-derived DNA library by different concentrations of human competitor DNA. Biotin-labeled chromosome 7 DNA inserts (20  $\mu\text{g/ml}$ ) were prehybridized for 20 minutes with human genomic DNA prior to hybridization with metaphase chromosome spreads and detection with FITC-labeled avidin. Genomic salmon DNA was added to each sample to adjust the final DNA concentration to 1.0 mg/ml (see the text for details). The arrows mark the target chromosome 7 and the arrowheads mark additional strong signals on non-7 chromosomes. All negatives printed were exposed and developed under identical photographic conditions.

Figure 2A shows prehybridization with 0  $\mu\text{g/ml}$  human competitor DNA.

Figure 2B shows prehybridization with 50  $\mu\text{g/ml}$  of human competitor DNA.

Figure 2C shows prehybridization with 100  $\mu\text{g/ml}$  of human competitor DNA.

Figure 2D shows prehybridization with 200  $\mu\text{g/ml}$  of human competitor DNA.

Figure 2E shows prehybridization with 1000  $\mu\text{g/ml}$  of human competitor DNA.

Figure 2F is the same as Figure 2E except that the metaphase spread is post-stained with DAPI.

Figures 3A-3D show the effect of pre-annealing time on the specificity and strength of the hybridization signal. Biotin-labeled chromosome 7 DNA inserts (20  $\mu\text{g/ml}$ ) were preannealed with 200  $\mu\text{g/ml}$  human competitor DNA for different times prior to hybridization to metaphase chromosomes.

Figure 3A shows preannealing for 0 minutes.

Figure 3B shows preannealing for 2 minutes.

Figure 3C shows preannealing for 5 minutes.

Figure 3D shows preannealing for 20 minutes.

Figure 4A show decoration of chromosome 1 in normal human lymphocytes. The signal of chromosome 1 was amplified by the sandwich technique of Pinkel et al. (1986).

Figure 4B shows decoration of chromosome 7 in normal human lymphocytes.

Figure 4C shows decoration of chromosome 4 in normal human lymphocytes.

Figure 4D shows decoration of chromosome 18 in normal human lymphocytes.

Figure 4E shows decoration of chromosome 13 in normal human lymphocytes. Only the chromosome 13 insert DNA pool shows significant cross-hybridization to other chromosomes after the prehybridization suppression step.

Figure 4F shows decoration of chromosome 20 in normal human lymphocytes. The detection of chromosome 20 was done with the entire chromosome library (including  $\lambda$  phage arms) and detected with avidin-alkaline phosphatase using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) as the enzyme substrate mixture.

Figures 5A-5F show chromosome domains in human lymphocyte nuclei delineated by preannealed chromosome library DNA inserts.

Figure 5A and Figure 5B show domains for chromosome 1. Hybridization to acetic acid-methanol fixed nuclei was detected by fluorescein isothiocyanate (FITC)-conjugated avidin.

Figure 5C and Figure 5D show domains for chromosome 7. Hybridization to acetic acid-methanol fixed nuclei was detected by fluorescein isothiocyanate (FITC)-conjugated avidin. A predominant staining of the centromere region is seen within the chromosome 7 domains, reflecting preferential hybridization of the chromosome 7-specific alphoid DNA repeat; a similar signal distribution on metaphase chromosomes was also observed in the particular experiment.

Figure 5E shows domains for chromosome 18. Hybridization to acetic acid-methanol fixed nuclei was detected by fluorescein isothiocyanate (FITC)-conjugated avidin.

Figure 5F shows domains for chromosome 18. Hybridization to acetic acid-methanol fixed nuclei was detected by alkaline phosphatase-conjugated avidin.

Figure 6A shows chromosomal in situ suppression (CISS) hybridization of chromosome 1 inserts to metaphase spreads of the TC 620 glioma cell line detected with FITC-avidin. Figure 6B is the same as Figure 6A except that the metaphase

spreads are post-stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). TC 620 show two apparently complete 1 chromosomes (small arrows in B) and two marker translocation chromosomes (arrowheads) specifically decorated by these inserts. One of the two marker chromosomes contains a 1p (lower left), the other a 1q arm (lower right); the 1p terminal (relatively GC rich region) in the two normal chromosomes and submetacentric marker is less completely delineated. Also, the 1q12 regions here show little decoration in contrast to most experiments. X950. Figure 6C shows chromosomal in situ suppression (CISS) hybridization of chromosome 1 inserts to metaphase spreads of the TC 593 glioma cell line detected with FITC-avidin.

Figure 6D is the same as Figure 6C except that the metaphase spreads are post-stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Typical TC 593 metaphase spreads show six specifically decorated chromosomes. Three acrocentric marker chromosomes all with truncation of 1p show particularly intense fluorescence of repeats that localize to 1q12 (arrows in C). In two of these, 1q arms appear to be complete, while a major deletion is obvious in the third (arrow in D). A fourth decorated chromosome (small arrowhead in C, D) again shows a major deletion of the distal part or 1q, but has retained an apparently complete 1p arm. A fifth submetacentric chromosome (large arrowhead) contains an apparently complete 1p arm; the DNA of its short arm is not identified. Note the similarity of this marker to one of the marker chromosomes of TC 620 (1p) described above. The sixth entirely decorated chromosome is an iso (1p) as demonstrated by DAPI-binding (open arrows). X 1200.

Figures 7A-7J show CISS hybridization of chromosome 4 library inserts detected with FITC-avidin.

Figure 7A shows interphase nuclei of TC 593. Note that the two apparently complete interphase domains are widely separated.

Figure 7B shows interphase nuclei of TC 593. Note that the two apparently complete interphase domains are close to each other.

Figure 7C shows interphase nuclei of TC 593. Note that the two apparently complete interphase domains are widely separated.

Figure 7D shows interphase nuclei of TC 620 showing four chromosome 4 interphase domains of largely different sizes.

Figure 7E shows metaphase spread of TC 593 showing two apparently complete 4 chromosomes, and a small decorated region (arrow) in a submetacentric chromosome. This marker with translocated 4 sequences was observed in about 30% of the spreads.

Figure 7F shows metaphase spread of TC 620 showing one apparently complete chromosome 4 and three translocation markers (t) containing different amounts of chromosome 4 material.

Figure 7G shows double hybridization of biotinylated chromosome 7 inserts and an aminoacetylfluorene (AAF)-modified 7-specific alphoid repeat. Chromosome 7 inserts depict five entirely decorated metaphase chromosomes. Four of them are complete 7 chromosomes, the fifth (arrow) is an iso (7p) (see Figure 3E).

Figure 7H shows the same field as G showing AAF-7 alphoid signals on only four decorated chromosomes; no signal is detected on the iso (7p).

Figure 7I shows an interphase nucleus of TC 593 showing five domains delineated by chromosome 7 inserts. The arrow represents the iso (7p) marker in interphase.

Figure 7J shows the same field as I showing that four of the domains are labeled by 7 alphoid probes.

Figure 8A shows CISS hybridization of library inserts of chromosome 7 to metaphase spreads of TC 620 glioma cells detected with FITC-avidin. x 875. Three apparently normal 7 chromosomes and an additional translocation chromosome containing 7 sequences are indicated by large arrowhead.

Figure 8B is the same as for Figure 8A except counter-stained with DAPI. DAPI-stained complete chromosomes are indicated by small arrowheads. Other studies (see the text) indicated a translocation of 7pter-q11 in the marker chromosome (large arrowhead).

Figure 8C shows CISS hybridization of library inserts of chromosome 18 to metaphase spreads of TC 620 glioma cells detected with FITC-avidin. Two apparently complete 18 chromosomes and a truncated minute chromosome (large arrowhead) are shown.

Figure 8D is the same as for Figure 8C except counter-stained with DAPI.

Figure 8E shows metaphase spread from pseudotetraploid TC 593 cells showing five chromosomes highlighted by 7 library inserts. The metacentric chromosome (m) represents the iso (7p) marker typical for this line (see also Figure 2G). Insert chromosomes (small arrows) show DAPI-stained normal and metacentric 7 chromosomes. The landmark band 7q21 and a block of constitutive heterochromatin at 7q11 are both prominent on the normal chromosome 7 insert (arrows) but not present on the marker chromosome. Instead both arms of the latter show a mirror-like staining pattern with a faint distal band at 7p21.

Figure 8F shows CISS hybridization of library inserts of chromosome 18 to metaphase spreads of TC 593 glioma cells detected with FITC-avidin. Four decorated 18 chromosomes are shown.

Figure 8G is the same as for Figure 8F except counter-stained with DAPI. Three decorated 18 chromosomes are clearly translocated.

Figure 9 is a summary chromosome idiogram of complete and aberrant chromosomes detected by CISS hybridization of library inserts of chromosome 1, 4, 7, 18 and 20 in glioma cell lines TC 620 (left) and TC 593 (right). G-bands (black) are shown with approximate breakpoints suggested by our data; the shaded areas with a wavy pattern are from other chromosomes that constitute part of the marker translocation chromosomes. The black dot beside two of the TC 620 translocated 4 segments indicates that the assignment of the chromosome 4 material is based on circumstantial evidence (e.g., size measurements). A small translocation of chromosome 18 material in ca. 20% of TC 593 metaphase spreads (+) also could not be further identified. Note the over-representation of 7p in both cell lines.

Figures 10A-10H show representative nuclei reflecting metaphase abnormalities in glioma cell lines (cf. Figs 1-3).

Figure 10A: Detection of the 7p translocation (t) in a prophase TC 620 nucleus. X 1,000.

Figure 10B: Detection of five well-separated chromosome 7 domains in interphase TC 593. X 1,240.

Figure 10C: Detection of two large and one very small 18 domains (indicated by arrowheads) in interphase TC 6. X 1,450.

Figure 10D: Detection of four chromosome 18 domains in interphase TC 593; one of these signals (arrows) appears smaller. X 1,450.

Figure 10E: Detection of four chromosomal 1 domains detected in interphase TC 620 (cf. Figure 1A, 1B). X 1,200.

Figure 10F: Detection of at least five chromosome 1 domains in interphase TC 593; one (arrow head) is appreciably smaller than the others. X 1,250.

Figure 10G: CISS hybridization of a metaphase spread of TC 593 with chromosome 18.

Figure 10H: The same as for 10G except counter-stained with DAPI. The technically poor metaphase spread still highlights four distinct chromosomes bearing 18 sequences. X 1,000.

Figures 11A-11E are graphic representations of the interphase and/or metaphase counts of chromosomes 7, 22 and 4 by CISS hybridization. Interphase counts were performed on 150 nuclei of well-hybridized preparations. For metaphase counts > 25 complete DAPI-stained spreads were evaluated.

Figure 11A: Counts of 7 specific alphoid repeats (white columns) compared to 7 library inserts (shaded columns) from interphase nuclei of phytohemagglutinin-stimulated human lymphocytes (46, XY).

Figure 11B: The same as for Figure 11A except from TC 620 interphase nuclei (7-specific alphoid repeats) and metaphase spreads (7 library inserts).

Figure 11C: The same as for Figure 11A except from TC 593 interphase nuclei (7-specific alphoid repeats) and metaphase spreads (7 library inserts).

For Figures 11 A-C: High stringency hybridization (see Materials and Methods) of 7 alphoid repeat was used to avoid cross-hybridization to other chromosomes. In cases of double hybridization with both 7 library inserts and alphoid repeat (shown in Figure 2 G-I) standard conditions with 50% formamide were sufficient to avoid cross-hybridization, possibly due to the presence of human competitor DNA.

Figure 11D: Counts of chromosome 22 (library inserts) in metaphase spreads of TC 620 (black columns) and TC 593 (shaded columns). For comparison, CISS hybridization was simultaneously performed with 7 library inserts in these experiments as an internal control (see C, D and Fig. 7).

Figure 11E: Interphase counts (white columns) and metaphase counts (shaded columns) compared in TC 593 hybridized with chromosome 4 inserts. Note the ratio of signal preparations 2:3 are the same in metaphase and interphase.

Figure 12 shows a TC 620 metaphase spread after double hybridization with inserts from chromosome 7 and 22 (both labeled with biotin and detected with avidin-FITC). Two strongly decorated 22 chromosomes (arrows), three complete 7 chromosomes and the metacentric marker chromosome containing 7pter-q11 are also seen.

Figure 13 is a graphic representation of the relative size of decorated normal and aberrant chromosomes 4, 7 and 18 in typical metaphase spreads (n=24) from glioma cell lines TC 593 and TC 620. Individual areas were normalized so that a complete chromosome is represented by an area of 1 (see legend to Table 1). The total added signals reflect the number of specific chromosome equivalents present. The white regions correspond to apparently normal chromosomes, the black regions indicate small

free chromosome segments entirely decorated by specific library inserts, and translocated segments are shaded. One of the three translocated 18 chromosomes in TC 593 represents a complete chromosome by this measurement (indicated by the black dot), while the two other translocations are slightly smaller, possibly due to the small sample size.

Figures 14A-14O show specific labeling of human chromosome 21 by CISS hybridization with biotinylated DNA probe sets.

Figure 14A: Hybridization of plasmid pPW519-1R (6 kb insert) to a normal lymphocyte metaphase spread. Signals are located on the termini of 21q (see DAPI-stained chromosomes in Inset) as verified by DAPI banding (not shown).

Figure 14B: Hybridization of the 94 kb plasmid pool probe set to normal human lymphocyte metaphase spread. The terminal band 21q22.3 is specifically labeled.

Figure 14C: The same as for Figure 14B except that hybridization was to normal human lymphocyte nuclei.

Figure 14D: Hybridization of the 94 kb probe set to trisomy 21 (47, +21) lymphocyte metaphase spreads.

Figure 14E: Hybridization of chromosome 21 library DNA inserts to trisomy 21 (47, +21) lymphocyte metaphase spreads. Three chromosomes 21 are entirely delineated by the library inserts; additional minor signals (see the text) are indicated by arrowheads (also in Figure 14G).

Figure 14F: Hybridization of chromosome 21 library DNA inserts to trisomy 21 (47, +21) lymphocyte interphase nuclei (compare with 14E).

Figure 14G: Hybridization of chromosome 21 library DNA inserts to trisomy 21 (47, +21) lymphocyte interphase nuclei.

Figure 14H: Hybridization of chromosome 21 library DNA inserts to trisomy 21 (47, +21) lymphocyte interphase nuclei.

Figure 14I: Hybridization of chromosome 21 library DNA inserts to trisomy 21 (47, +21) lymphocyte interphase nuclei.

Figure 14J: Hybridization of chromosome 21 library DNA inserts to trisomy 21 (47, +21) lymphocyte interphase nuclei.

Figure 14K: Hybridization of the 94 kb probe set to chorionic villi (CV) cell interphase nuclei.

Figure 14L: Hybridization of the 94 kb probe set to CV cell metaphase spreads.

Figure 14M: Hybridization of the 94 kb probe set to CV cell metaphase spreads.



sufficiently to produce labeled probe DNA hybridizable [essentially] specifically to the target chromosomal DNA.

29. (Amended) The method of claim 22, for simultaneous detection of more than one chromosome, or region, thereof, wherein the probe DNA for each chromosome, or region thereof, is labeled with or labelable with a different fluorophore or a different combination of fluorophores to yield optically distinguishable signals.
30. (Amended) A method of *in situ* hybridization for distinguishably labeling individual human chromosomes, or regions thereof, in interphase cells comprising:
- a) providing labeled probe DNA for each chromosome, or region thereof, to be visualized, each probe having sequences specifically hybridizable to target chromosomal DNA, but having repetitive sequences which cross-hybridize to non-target chromosomal DNA and each probe DNA being labeled with or labelable with a different fluorophore or a different combination of fluorophores to generate optically distinguishable signals; and
  - b) combining
    - i) the labeled probe DNA for each chromosome, or region thereof;
    - ii) a competitor DNA containing the repetitive sequences; and
    - iii) a sample of interphase cells, or nuclei thereof, treated to render chromosomal DNA therein available for hybridization with the labeled probe DNA,
- under hybridization conditions wherein cross-hybridization between repetitive sequences in the probe DNA and the sample is sufficiently suppressed to allow the probe DNA to hybridize [essentially] specifically to the target chromosomal DNA, to thereby distinguishably label individual chromosomes.
31. (Amended) A method of *in situ* hybridization for visualizing an individual human chromosome, or region thereof, in interphase cells comprising

- a) providing biotinylated DNA fragments smaller than 500 nucleotides derived from a DNA library of a target chromosome, or region thereof, for visualization;
- b) combining biotin-labeled DNA fragments with fragments of total genomic DNA having the same size distribution as the labeled fragments and incubating the mixture under initial conditions that allow denaturation of the fragments and, subsequently, under conditions that promote annealing of fragments containing repetitive sequences but not fragments containing chromosome-specific sequences, to produce probe DNA [essentially] specific for the target chromosome;
- c) combining the probe DNA with a [cellular preparation] sample of interphase cells, or nuclei thereof, treated to render target chromosomal DNA available for hybridization with the probe DNA, under conditions which allow the probe DNA to hybridize to the target chromosomal DNA; and
- d) visualizing the individual human chromosome, or region thereof, by detecting the probe DNA by incubating the preparation with an avidin conjugated fluorophore and the detection of the fluorescent signal.

32. (Amended) A method of *in situ* hybridization for simultaneously visualizing individual human chromosomes, or regions thereof, in interphase cells comprising

- a) providing labeled probe DNA for each chromosome, or region thereof, to be visualized, each probe DNA having sequences specifically hybridizable to target chromosomal DNA, but having repetitive sequences which cross-hybridize to non-target chromosomal DNA and each probe DNA being labeled with or labelable with a different fluorophore or a different combination of fluorophores to generate optically distinguishable signals;
- b) combining
  - i) the labeled probe DNA for each chromosome, or region thereof;
  - ii) a competitor DNA containing the repetitive sequences; and
  - iii) a sample of interphase cells, or nuclei thereof, treated to render chromosomal DNA therein available for hybridization with the labeled probe DNA, under hybridization conditions wherein cross-hybridization between repetitive sequences in the probe DNA and the sample is sufficiently suppressed

to allow each probe DNA to hybridize [essentially] specifically to the target chromosomal DNA; and

c) detecting the optically distinguishable signals generated by each probe DNA to simultaneously visualize each chromosome.

33. (Amended) A method according to claim 32, wherein the detecting step includes the step of

a) generating one or more digital images of the hybridized chromosomal DNA; and

b) visually emphasizing those portions of the digital images which represent optically distinguishable signals associated with a [fluorophor] fluorophore or combination thereof.

35. (Amended) A method according to claim 34, wherein the visually emphasizing step includes the steps of generating an image of the hybridized chromosomal DNA by

a) assigning a respective color to each [fluorophor] fluorophore; and

b) substituting, for those portions of the digital images which represent optically distinguishable signals associated with the [fluorophors] fluorophores, or combinations thereof, respective colors assigned thereto.

### REMARKS

The above amendments and following remarks are directed to rejections which remained outstanding in an Action (Paper No. 31) dated December 23, 1993 issued in U.S. Serial No. 07/837,664, of which this application is a File Wrapper Continuation.

The Brief Description of the Drawings in the specification has been amended to correctly identify the figures as suggested by the Examiner in the Action of December 23, 1993 issued in U.S. Serial No. 07/837,664. The amendment of the Brief Description of the Drawings submitted herewith is intended to replace the amendment of the Brief Description of the Drawings filed on September 20, 1993 in U.S. Serial No. 07/837,664.

Claims 19-35 are pending in this application (USSN 08/312,429). The claims have been amended to more clearly recite and distinctly claim the subject matter that Applicants consider to be their invention. The claims have been amended in the interest of expediting the prosecution of this application and these amendments should in no way be construed as an acquiescence to any of the Examiner's rejections issued in U.S. Serial No. 07/837,664. Applicants reserve the right to refile claims in this or another application.

Claims 19, 21, 30, 31 and 32 have been amended to remove the word "essentially" in the phrase "essentially specific." As amended, the claims are directed to methods in which a DNA probe hybridizes "specifically" to a target chromosomally DNA. This amendment addresses and overcomes the rejection of these claims for indefiniteness under 35 U.S.C. § 112, second paragraph. Accordingly, Applicants respectfully request that this rejection be withdrawn.

Claims 30, 31 and 32 have been amended to indicate that the hybridization method is performed in "interphase cells, or nuclei thereof." Support for this amendment can be found throughout the specification, for example, at page 13.

Claims 29, 30 and 32 have been amended to indicate that the probes are "labeled with or labelable with" a different fluorophore or combination of fluorophores. Support for this amendment can be found throughout the specification, for example at page 15, lines 29-31, page 19, lines 2-14 and page 23, lines 13-19.

Claims 33 and 35 have been amended to correct certain informalities.

#### Rejection of Claims 19-25, 27-28 and 31 under 35 U.S.C. § 103

Claims 19-25, 27-28 and 31 remained rejected under U.S.C. § 103 as being unpatentable over Landegent et al. in view of Devilee et al for reasons set forth in Office Actions issued in U.S. Serial No. 07/837,664 dated March 18, 1993 and December 23, 1993.

The invention is directed to a method for detecting target chromosomal DNA *in situ* in interphase cells. The method involves combining a labeled probe DNA and competitor DNA, incubating the probe and competitor DNA with interphase cells, or nuclei thereof, under hybridization conditions and detecting the labeled probe DNA to thereby detect the target chromosomal DNA. The labeled probe DNA used in the method is characterized by containing sequences specifically hybridizable to the target chromosomal DNA and, additionally, repetitive sequences which hybridize to non-target chromosomal DNA. The competitor DNA used in the method contains the repetitive sequences which hybridize to non-target chromosomal DNA. Thus, in the claimed method, hybridization of the repetitive sequences in the DNA probe to non-target chromosomal DNA in interphase cells is suppressed by the competitor DNA.

The proper inquiry under section 103 is first whether the prior art would have suggested the claimed invention to one having ordinary skill in the art at the time the invention was made and that there was a reasonable expectation of success for the claimed invention in view of the prior art. Under section 103, "[b]oth the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure" (*Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.* 927 F.2d 1200,1207, 18 USPQ2d 1016 (Fed. Cir. 1991) quoting *In re Dow Chemical Co.*, 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed Cir. 1988). Additionally, when a combination of references are used to establish a *prima facie* case of obviousness, it is necessary for the Examiner to present evidence that one having ordinary skill in the art would have been motivated to combine the teachings in the applied references in the proposed manner to arrive at the claimed invention. See, e.g., *Carella v. Starlight Archery*, 804 F.2d 135, 231 USPQ 644 (Fed. Cir. 1986); and *Ashland Oil, Inc. v. Delta Resins and Refractories, Inc.*, 776 F.2d 281, 227 USPQ 657 (Fed. Cir. 1985).

Landegent et al. teach *in situ* hybridization in metaphase cells using as probes one or more cosmids of the human Tg gene. Landegent et al. (at page 367, column 2, second paragraph of the Results section) describe the probes used in their method as follows:

four different randomly chosen cosmids of the human Tg gene (of which the chromosomal localization is known, Baas et al. 1985; Landegent et al. 1985b) were used (Van Ommen et al. 1983; Baas et al. 1986)....These cosmids have not been characterized in the actual number and type of repeats present, but all gave a strong overall staining when hybridized to total human DNA under standard conditions.

Thus, the DNA probes of Landegent et al. contain unique sequences that hybridize to a target chromosomal DNA (i.e., the Tg gene) but also contain repetitive sequences that hybridize to non-target chromosomal DNA, as does the DNA probe of the method of claim 19 (and dependent claims thereof). Furthermore, in the method of Landegent et al., competitor DNA that contains the repetitive sequences is included in the hybridization procedure to inhibit hybridization of the repetitive sequences to non-target chromosomal DNA, thereby allowing for specific hybridization of the unique sequences of the DNA probe to the target chromosomal DNA. Importantly, the method of Landegent et al. differs from the claimed method in that it is performed only in *metaphase* cells not *interphase* cells, or nuclei thereof.

Accordingly, the issue with regard to the obviousness of the claimed method is whether one of ordinary skill in the art at the time the invention was made, in view of the cited references, would have been motivated to apply the *in situ* suppression hybridization method taught by Landegent et al. to interphase cells, or nuclei thereof, and whether there was a reasonable expectation of success in applying the method to interphase cells, or nuclei thereof.

When Landegent et al. itself is examined for the necessary motivation and reasonable expectation of success in applying the method described therein to interphase

cells, it is apparent that Landegent et al. teach that the method described therein is *not* applicable to interphase cells. In particular, Landegent et al. (at page 369, column 2, last paragraph) state:

The method described here can extend the applicability of non-radioactive procedures since it elegantly bypasses the lower sensitivity problem, obviating subcloning of a minimal amount of unique parts required.

**When, in time**, the sensitivity has reached a level allowing the detection of small (1-2 kb) single-copy sequences on metaphase chromosomes on a routine basis, the described procedure may still retain its attractiveness for several purposes. For example: ...

2. Detection of chromosomal aberrations in prenatal diagnosis, for example a trisomy, in *interphase nuclei* in microscopic slides (Cremer et al. 1986) or through hybridization in suspension and flow cytometry. In these cases it would be favorable to use a whole panel of chromosome-specific cosmid instead of a small unique probe or a cloned alphoid sequence to ensure strong hybridization signals with great specificity, for a more reliable diagnosis [emphasis added].

The above-quoted passage contains the only reference to interphase cells, or nuclei thereof, in Landegent et al. and it is entirely future-oriented ("when, in time..."). Thus, Landegent et al. clearly teach that the sensitivity of their method is not sufficient for application to interphase cells, or nuclei thereof, at least in current form.

Accordingly, in view of the teachings of Landegent et al. alone, one of ordinary skill in the art at the time the invention was made would not have been motivated to apply the method described therein to interphase cells because Landegent et al. teach that their method, in current form, is not applicable to interphase cells. Similarly, in view of the teachings of Landegent et al. alone, one of ordinary skill in the art at the time the invention was made would not have had a reasonable expectation of success since Landegent et al. teach that the method described therein is not sensitive enough for use with interphase cells.

For the forgoing reasons, the claimed methods are nonobvious over Landegent et al. alone. We next turn to the combined teachings of Landegent et al. and Devilee et al. The Examiner rejected claims 19-25 and 27-28 under 35 U.S.C. § 103 as being unpatentable over Landegent et al. in view of Devilee et al. because

one of ordinary skill in the art at the time the invention was made would have been motivated to use the method taught by Landegent et al. in interphase cells as taught by Devilee et al. because Devilee et al. suggest the advantages of hybridization with interphase cells and further one of ordinary skill in the art at the time the invention was made would have further had a reasonable expectation of success because Devilee et al. taught that *in situ* hybridization could be accomplished with interphase cells [see page 7 of the Office Action dated December 23, 1993 for USSN 07/837,6664].

As discussed previously, when an obviousness rejection is based on a combination of references, it is necessary for the Examiner to present evidence that one having ordinary skill in the art would have been motivated to combine the teachings of the applied references in the proposed manner to arrive at the claimed invention. See e.g., *Carella*, cited *supra*, *Ashland*, cited *supra*. Moreover, there must have been a reasonable expectation of success in making the claimed invention in view of the teachings of the combined references. For reasons described in detail in the following subsections, neither the requisite motivation to combine the teachings of the cited references nor the reasonable expectation of success in making the claimed invention are provided by the cited references.

A. Devilee et al. Do Not Provide the Motivation to Combine the Teachings of Landegent et al. and Devilee et al.

Devilee et al. describe an analysis of seven primary breast tumors by *in situ* hybridization to detect chromosomal abnormalities therein. The *in situ* hybridization



method taught by Devilee et al. uses interphase nucleic and chromosome specific repetitive DNA probes. More specifically, the probes of Devilee et al. are composed of "repetitive DNA sequences located predominantly in the (peri)centromeric region of one particular chromosome" (see page 5826, first sentence of Discussion). These repetitive DNA sequence probes "under proper hybridization conditions, reveal only their cognate chromosomes as distinct spots in an interphase nucleus" (see page 5826, first sentence of Discussion). Thus, the probes of Devilee et al. differ markedly from the probes of Landegent et al. More specifically, the probes of Devilee et al. contain repetitive sequences that specifically hybridize to target chromosomal DNA whereas the probes of Landegent et al. contain both unique sequences that specifically hybridize to the target chromosomal DNA of interest and repetitive sequences that hybridize to non-target chromosomal DNA.

The Examiner relies upon Devilee et al. for providing the necessary motivation to combine the teachings of Landegent et al. and Devilee et al. In particular, the Examiner points to the statement in Devilee et al. that " 'interphase cytogenetics' will prove to be a powerful tool in the study of cytogenetic heterogeneity in solid tumors" (see Devilee et al. page 5825, column 1, last two sentences of the Introduction) as providing the necessary motivation to apply the *in situ* hybridization method of Landegent et al. to interphase cells. Whatever motivation this statement may have provided to the ordinarily skilled artisan must be evaluated within the context in which the statement was made. The cited statement is set forth in the Introduction of Devilee et al. (at page 5825). The first paragraph of the Introduction describes problems in karyotyping solid tumors previously experienced in the field. Specifically, Devilee et al. state that

[k]aryotyping procedures of solid tumors normally present difficulties such as a low mitotic index, reluctance of the cells to grow *in vitro* and poor quality metaphase figures, in which complex chromosomal rearrangements are difficult to identify (1). These drawbacks have hampered the cytogenetic characterization of extended series of solid

tumor cases. As a consequence, it has been difficult to establish representative chromosomal aberrations or to gain insight into the existence of cytogenetic inter- or intratumor heterogeneity

Thus, in this paragraph, Devilee et al. were setting up a problem in cytogenetic characterization of solid tumors, namely that metaphase analysis was difficult to perform with solid tumors because the cells had a low mitotic index, did not grow well *in vitro* and gave poor quality metaphase figures.

In the second paragraph of the Introduction, Devilee et al. go on to describe how they have addressed this problem in tumor karyotyping. Specifically, Devilee et al. state

It has been demonstrated (2-4) that ***ISH [in situ hybridization] of chromosome specific repetitive DNA probes***, the target sequences of which lie mainly in the (peri)centromeric region, can reveal those chromosome regions as distinct clusters of signal or spots within the interphase nucleus. Thus, ***this method***, which is fast and relatively easy, allows the counting of the number of target sites in a large number of tumor cells harvested directly from the primary cancer, without any cultivation step. We show here that the number of spots/nucleus (S/N) indicates chromosomal ploidy and/or aberrations. With regard to cellular and cytogenetic heterogeneity of solid tumors, ***the technique*** importantly allows identification of subpopulations of karyotypically aberrant cells. Analysis of seven primary breast tumors indicate that "interphase cytogenetics" will prove to be a powerful tool in the study of cytogenetic heterogeneity in solid tumors [emphasis added].

Thus, in this paragraph, Devilee et al. describe how their present study has addressed the previous problems with metaphase analysis of solid tumors. More specifically, in this paragraph, Devilee et al. describe their own successful application of ***ISH with chromosome specific repetitive DNA probes*** to tumor karyotyping in interphase nuclei. When read in context, it is apparent that the statement by Devilee et al. that " 'interphase cytogenetics' will prove to be a powerful tool in the study of cytogenetic heterogeneity in solid tumors" refers specifically to ***ISH with chromosome specific repetitive DNA probes*** as used by Devilee et al.

Devilee et al. do not teach or suggest that ISH with other types of DNA probes could be performed with interphase cells or nuclei. In particular, Devilee et al. do not teach or suggest that ISH with probes containing both unique sequences that hybridize to target chromosomal DNA and repetitive sequences that hybridize to non-target chromosomal DNA (as used by Landegent et al.) could be performed with interphase cells or nuclei. Rather, the discussion of "interphase cytogenetics" in Devilee et al. focuses solely on the use of ISH with DNA probes having repetitive sequences that hybridize to target chromosomal DNA. Thus, there is nothing in the teachings of Devilee et al. that would have directed one of ordinary skill in the art at the time the invention was made to apply the method of Landegent et al., which uses a completely different type of probe, to interphase cells or nuclei.

Recently, the Board of Patent Appeals and Interferences addressed the issue of the sufficiency of motivation required for combining the teachings of various references (*Ex parte Levengood*, 28 USPQ2d 1300 (BPAI 1993)). Specifically, the Board stated that "an Examiner cannot establish obviousness by locating references which describe various aspects of a patent applicant's invention without also providing evidence of the motivating force which would *impel* one skilled in the art to do what the patent applicant has done" (emphasis added). *Ex parte Levengood, supra*, at page 1302. Applying this standard to the quoted statement in Devilee et al., the Examiner has not met the burden of providing evidence of a motivating force sufficient to have *impeled* a person of ordinary skill in the art at the time the invention was made to apply the method of Landegent et al. to interphase cells, or nuclei thereof.

B. Landegent et al. Teach Away From Combining the Teachings of Landegent et al. and Devilee et al.

Not only does Devilee et al. not provide the motivation to combine the teachings therein with the teachings of Landegent et al., but additionally Landegent et al. teach away from combining the teachings of Landegent et al. and Devilee et al. In particular, with regard to application of the ISH suppression method described therein to interphase cells (once the sensitivity has been increased), Landegent et al. (at page 369, column 2, last paragraph) state that

In these cases [i.e., detection of chromosomal aberrations in interphase nuclei], it would be favorable to use a whole panel of chromosome-specific cosmids *instead of* a small unique probe or *a cloned alphoid sequence* to ensure strong hybridization signals with great specificity, for a more reliable diagnosis [emphasis added].

Thus, Landegent et al. teach that cloned alphoid sequences are not preferred for use as probes in their method to detect chromosomal aberrations in interphase cells or nuclei.

However, *the ISH method of Devilee et al. uses cloned alphoid sequences as DNA probes*. Devilee et al. state that their probes "belong to a class of repetitive sequences referred to as  $\alpha$  satellite (7, 8)" (see page 5825, second column, Material and Methods section, under the heading "DNA Probes"). These  $\alpha$  satellite sequences are also referred to in the art as alphoid sequences. For example, reference number 7, which Devilee et al. cite to describe their DNA probes, is entitled "Two subsets of human *alphoid repetitive DNA* show distinct preferential localization in the pericentric regions of chromosomes 13, 18 and 21" (see page 5829, second column, reference 7).

Accordingly, since Landegent et al. teach that cloned alphoid sequences are not preferred for use as DNA probes in their method to detect chromosomal aberrations in interphase cells yet Devilee et al. teach an ISH method which utilizes alphoid sequences

as DNA probes, Landegent et al. actually teach away from combining the teachings therein with the teachings of Devilee et al.

C. Landegent et al. and Devilee et al. Do Not Provide a Reasonable Expectation of Success in Making the Claimed Invention

For the reasons discussed in the previous sections, one of ordinary skill in the art at the time the invention was made would not have been motivated to combine the teachings of Landegent et al. and Devilee et al. in the proposed manner to arrive at the claimed invention. However, even if one were to assume that the requisite motivation to combine the teachings of the references were present, one of ordinary skill in the art at the time the invention was made would not have had a reasonable expectation of success in making the claimed invention in view of the teachings of the cited references for the following reasons.

At the time the invention was made, Landegent et al. taught that their ISH suppression method, while useful in metaphase cells, is not applicable to interphase cells in current form. In particular, as previously discussed, Landegent et al. taught that their method might be useful in interphase cells only "when, in time, the sensitivity has reached a level allowing the detection of small (1-2 kb) single-copy sequences on metaphase chromosomes on a routine basis" (see page 369, column 2, last paragraph). Thus, Landegent et al. taught that the sensitivity of their method would have to be improved in metaphase cells before it would be applicable to interphase cells or nuclei. However, at the time the invention was made, Devilee et al. provided no guidance to the skilled artisan as to how the sensitivity of the method of Landegent et al. could be improved so that it could be used successfully in interphase cells or nuclei. Absent such guidance, one of ordinary skill in the art would not have had a reasonable expectation of success in applying the method of Landegent et al. to interphase cells, or

nuclei thereof, since Landegent et al. taught that their method would be applicable to interphase cells or nuclei only "when, in time" the sensitivity of the method had been improved.

The Examiner argues that one of ordinary skill in the art would have had a reasonable expectation of success in applying the method of Landegent et al. to interphase cells or nuclei simply because "Devilee et al. taught that *in situ* hybridization could be accomplished with interphase cells" (see page 7 of the Office Action of December 23, 1993 in USSN 07/837,663). Applicants contend that this is too broad an interpretation of the teachings of Devilee et al. More precisely, Devilee et al. teach *in situ* hybridization with interphase cells ***only using chromosome specific repetitive DNA probes*** (i.e., alphoid sequence probes). Thus, at the time the invention was made, Devilee et al. provided no teaching or guidance to the skilled artisan as to how ISH could have been performed with interphase cells, or nuclei thereof, using other types of DNA probes. Moreover, as previously discussed, Landegent et al. specifically state that the probes taught by Devilee et al. (i.e., alphoid sequences) would not be preferred for use in their ISH method for detection of chromosomal abnormalities in interphase cells or nuclei. Accordingly, at the time the invention was made, the ordinarily skilled artisan would not have had a reasonable expectation of success in applying the ISH suppression method of Landegent et al. to interphase cells as cells taught by Devilee et al. since Landegent et al. teaches away from using the type of DNA probe used by Devilee et al. and Devilee et al. provides no guidance for using other types of DNA probes in interphase cells.

D. Conclusion

In summary, a *prima facie* case of obviousness has not been established for the claimed method in view of the combined teachings of Landegent et al. and Devilee et al. for the following reasons:

1) Devilee et al. fails to provide the necessary motivation for combining the teachings of the two references because the statement in Devilee et al. discussing the power of "interphase cytogenetics" refers only to ISH with chromosome specific repetitive DNA (i.e., alphoid sequence) probes. Devilee et al. does not teach or suggest applying ISH to interphase cells or nuclei with other types of DNA probes, in particular the probes of Landegent et al.;

2) Landegent et al. teaches away from combining the teachings of the cited references, since Landegent et al. teach that alphoid sequences are not preferred for use as probes in their ISH method in interphase cells, or nuclei thereof, yet Devilee et al. only teach ISH in interphase nuclei using alphoid sequences as probes; and

3) Even if there were a motivation to combine the teachings of Landegent et al. and Devilee et al., there would have been no reasonable expectation of success for the claimed method in view of these teachings, since Landegent et al. teach that the sensitivity of the ISH suppression technique described therein is not sufficient for application to interphase cells or nuclei but Devilee et al. provide no guidance as to how one skilled in the art could improve the sensitivity of the method of Landegent et al. so that it would be successful in interphase cells. Additionally, Landegent et al. teach that alphoid sequences are not preferred for use as DNA probes in their method yet Devilee et al. teach ISH with interphase cells or nuclei only using alphoid sequences as DNA probes and provide no guidance for use of other types of DNA probes in interphase cells or nuclei.

Rejection of Claims 26, 29-30 and 32-35 under 35 U.S.C. § 103

All other pending claims (26, 29-30 and 32-35) remained rejected under 35 U.S.C. § 103 as being unpatentable over Landegent et al. in view of Devilee et al. and other secondary references or in view of Landegent et al. and other secondary references.

Each of these claims (as originally filed or as amended herein) claim a method of *in situ* hybridization in which a labeled probe DNA containing sequences specifically hybridizable to target chromosomal DNA and, additionally, repetitive sequences which hybridize to non-target chromosomal DNA, is combined with competitor DNA that contains the repetitive sequences and used to label or detect the target chromosomal DNA in *interphase cells, or nucleic thereof*.

The methods of claims 26, 29-30 and 32-35 are nonobvious over the teachings of Landegent et al. alone or in combination with Devilee et al. for the reasons described in detail above for rejection of claim 19-25 and 27-28 under 35 U.S.C. § 103 as being unpatentable over Landegent et al. in view of Devilee et al. The additional secondary references relied upon by the Examiner in the rejections of claim 26, 29-30 and 32-35 (i.e., Hames et al., Hopman et al. or Viegas-Pequignot et al.) do not make up for the deficiencies in Landegent et al. alone or in combination with Devilee et al. Hames et al. is relied on for teaching that short nucleic acid fragments (e.g., 50-150 nucleotides) are preferred for *in situ* hybridization (see page 8 of the Office Action dated December 23, 1993 in USSN 07/837,664). Hopman et al. is relied on for teaching labeling of two different DNA probes with distinguishable labels to allow for simultaneous detection of two different target DNA sequences (see page 7 of the Office Action dated March 18, 1993 in USSN 07/837,664). Viegas-Pequignot et al. is relied upon for teaching detection of *in situ* hybridization by intensified-fluorescence digital imaging microscopy (see page 10 of the Office Action dated March 18, 1993 in USSN 07/837,664).



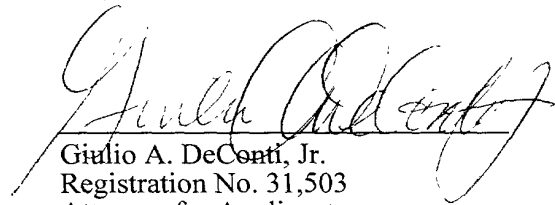
Accordingly, none of the additional secondary references relied upon by the Examiner provide the necessary motivation to apply the method of Landegent et al. to interphase cells or to combine the teachings of Landegent et al. and Devilee et al. to arrive at the claimed invention, nor do the additional secondary references provide the necessary reasonable expectation of success for the claimed methods.

### **SUMMARY**

In view of the above-described claim amendments and remarks, claims 19-35 are in condition for allowance. Accordingly, Applicants request reconsideration and withdrawal of the remaining rejections.

If a telephone conversation with Applicants' Attorney would expedite prosecution of the above-identified application, the examiner is urged to call the undersigned at (617) 227-7400.

Respectfully submitted,



Giulio A. DeConti, Jr.  
Registration No. 31,503  
Attorney for Applicants

LAHIVE & COCKFIELD  
60 State Street  
Boston, MA 02109

Dated: January 23, 1995

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: David C. Ward, Peter Lichter, Thomas Cremer,  
Laura Manuelidis, Thomas Ried and Antonio Baldini

File-wrapper-continuation of U.S. Serial No. 07/271,609  
filed November 15, 1988

Title: DELINEATION OF INDIVIDUAL HUMAN CHROMOSOMES  
IN METAPHASE AND INTERPHASE CELLS BY IN SITU  
SUPPRESSION HYBRIDIZATION

Attorney's Docket No.: IGI-001CN

\*\*\*\*\*  
I hereby certify that this correspondence is being deposited  
with the United States Postal Service in an envelope as  
"Express Mail Post Office to Addressee" mailing label number  
RB12901217X service under 37 CFR 1.10 addressed to: Box FWC,  
The Honorable Commissioner of Patents and Trademarks,  
Washington, D.C. 20231 on the date set forth below.

February 14, 1992  
Date of Signature  
and of Mail Deposit

By

  
Carlos R. Bothwell

\*\*\*\*\*

AMENDMENT

Box FWC  
The Honorable Commissioner  
of Patents and Trademarks  
Washington, D.C. 20231

Dear Sir:

Please amend patent application Serial No. 07/271,609  
filed November 15, 1988 as follows:

In the Specification

Please add new Figure 15 submitted herewith.

Page 12, before the heading "Detailed Description of the Invention", insert:

---Figure 15 (A-C) shows hybridization signals from centromeric repeat probes on metaphase chromosomes from a normal male. The labeling combinations used are given in Table 5. The images were taken separately with the appropriate filters and pseudocolored.

(A) Image taken with the fluorescein filter, displaying the fluorescein-11-dUTP-labeled probes for the centromeres of chromosomes 8, 11, 12 and 18. The arrowheads indicate the centromere for chromosome 12, which was singly labeled with fluorescein-dUTP. The arrows show the centromere of chromosome 8, which was labeled with a triple combination.

(B) Detection of the dig-labeled probes with the rhodamine-specific filter. The centromeres of chromosomes 7 (arrowheads), 8 (arrows), 9 and 18 reveal hybridization signals.

(C) Using the infrared filter combination, the biotinylated probes that were detected with streptavidin conjugated to the infrared dye Ultralite 680 are shown. Chromosomes 3 (arrowheads), 8 (arrows), 9 and 11 were detected.

(D,E) Independently acquired gray scale images were merged and pseudocolored, resulting in seven differentially colored centromeric sequences on metaphase chromosomes (D) and in an interphase nucleus (E). DAPI was used as a DNA counterstain.

(F) Example of combinatorial labeling of chromosome-specific libraries with PCR. The libraries for chromosomes 1, 2, 4, 8, 14 and X were labeled singly or combinatorially (see Table 2) and pseudocolored in green, pink, yellow, white, orange and red respectively.

(G) PCR-labeled chromosome-specific libraries were used for detection of a t(2;14) translocation. The library for chromosome 2 was labeled with biotin and detected with avidin fluorescein; the chromosome 14 library was labeled with dig and detected with anti-dig rhodamine. Both translocation chromosomes are clearly visible (arrowheads).

(H) Combinatorial labeling of cosmid and phage clones by nick-translation. Single gene probes for six different chromosomes were hybridized simultaneously. The probes and the labeling combinations are described in Materials and Methods. Chromosomes 5, 6, 8, 11, 21 and X show hybridization signals.

(I) Combinatorial labeling of six cosmid clones specific for chromosome 5. The differentially pseudocolored probes label six loci on this chromosome simultaneously.

(J) Hybridization of the chromosome 5 specific probes to an interphase nucleus. The order of the cosmid clone is maintained.---

Page 79, before the heading "Equivalents", insert:

---Example III: Simultaneous Visualization of Seven Different DNA Probes by In Situ Hybridization Using Combinatorial Fluorescence and Digital Imaging Microscopy

## MATERIALS AND METHODS

Human metaphase chromosomes were prepared by standard procedures. Prior to in situ hybridization, slides were washed in 1x phosphate-buffered saline (5 min; room temperature) and dehydrated through an ethanol series (70%, 90% and 100%; 5 min each). Slides were stored at -70°C with Drierite powder.

### DNA Probes

The following chromosome-specific  $\alpha$  satellite DNA clones were used: pBS10.7AE0.6 (Baldini, unpublished), chromosome 3; p7tet (Waye, J.S. et al. (1987) Mol. Cell. Biol. 7:349-356), chromosome 7; pMR9A (Rocchi, M. et al. (1991) Genomics 9:517-523), chromosome 9; pBR12 (Baldini, A. et al. (1990) Am. J. Hum. Genet. 46:784-788), chromosome 12. p $\alpha$ H2 (chromosome 18) and p $\alpha$ H5 (chromosome 8) were cloned in our laboratory, while pRB2 (chromosome 11) was a gift of Dr. M. Rocchi (Bari, Italy). The chromosome specific plasmid libraries (Collins, C. et al. (1991) Genomics, in press) were a gift of Dr. J. Gray (Livermore, CA). The following cosmid and phage clones were used: cpt1, mapping to Xp21 (Ried, T. et al. (1990) Hum. Genet. 85:581-586); c-myc, mapping to 8q24 (Ried T. et al. (1991) Genes Chromosom Cancer, in press); c512, mapping to 21q22 (Lichter, P. et al. (1990) in Molecular Genetics of Chromosome 21 and Down Syndrome, ed. Patterson, D., Alan R. Liss, New York, NY, pp. 69-78); cosmid clone 26, mapping to 5q32 (unpublished data); cosSB1, mapping to 6p21 (Srivastava, R. et al. (1986) Trans. Assoc. Am. Physicians, Vol. XCIX); cosmid K40, mapping to 11p15 (Lichter, P. et al. (1990) Science 247:64-69). The cosmid clones specific for chromosome 5 (clones 26, 29, 56, 58, 92 and 121) were provided by Dr. Greg Landes (Integrated Genetics, Inc., Framingham, MA) and previously mapped by Jennifer Lu (personal communication).

DNA was prepared according to standard techniques (Sambrook, J. et al. (1989) Molecular cloning: a laboratory manual, Cold Spring Harbor Lab., Cold Spring Harbor, NY).

#### Probe Labeling

PCR's were performed using 10 ng of aliphoid DNA clones or 100 ng of chromosome-specific libraries as template. Preferential amplification of insert DNA was achieved by using primers flanking the polylinker of each plasmid vector. T3 and T7 primers were used for the pBS vector, M13 forward and M13 reverse primers were used for pUC and pCR1000 vectors (all at a final concentration of 1  $\mu$ M). PCR was carried out in 1.5 mM  $MgCl_2$ /10 mM Tris-HCl/50 mM KCl/0.001% gelatin/1.25 units of Tag polymerase (AmpliTag; Perkin-Elmer/Cetus) in a total volume of 50  $\mu$ l (10  $\mu$ l when fluorescein-11-dUTP was used due to the limited amount of this reagent). The dNTP concentrations used in the PCR-labeling reactions are listed in Table 5. The highest concentration of modified nucleotides used was 75  $\mu$ M. However, dinitrophenol (DNP)-11-dUTP at a concentration  $>37.5$   $\mu$ M strongly reduced the amplification efficiency (data not shown). When DNP-11-dUTP was used for combinatorial labeling, the concentrations were the same as for fluorescein-11-dUTP. The modified nucleotides were obtained from Boehringer Mannheim (digoxigenin (dig)-11-dUTP, fluorescein-11-dUTP), Sigma (Bio-11-dUTP) and Novagen (Madison, WI) (DNP-11-dUTP). The thermocycling was performed with a commercially available machine (Ericomp, San Diego). After an initial denaturation at 95°C for 3 min, 32 cycles of PCR were carried out with denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 4 min (last cycle, 10 min). PCR products from the chromosome libraries were treated with DNase I to obtain an average fragment size of about 250 base pairs (bp) and were separated from free nucleotides by Sephadex G50 spin

column. Cosmid and phage clones were labeled by standard nick-translation reactions. The final concentration of the modified nucleotides and the DNA clones used in these reactions were as follows: Bio, 50  $\mu$ M (cosSB2, clone 58); dig, 40  $\mu$ M (K40, clone 121); DNP, 40  $\mu$ M (c512, clone 92); Bio/dig, 20  $\mu$ M/30  $\mu$ M (cpt1, clone 56); Bio/DNP, 20  $\mu$ M/30  $\mu$ M (c-myc, clone 29) and dig/DNP, 20  $\mu$ M/30  $\mu$ M (clones 28 and 26).

Table 5. Labelling of Seven Centromere Probes

	dNTP, $\mu$ M						
	AE.06	p7tet	pBR12	pMR9A	pRB2	p $\alpha$ H2	p $\alpha$ H5
Bio-11-dUTP	75			37.5	37.5		25
dig-11-dUTP		75		37.5		37.5	25
FITC-11-dUTP			75		37.5	37.5	25
TTP	225	225	225	225	225	225	225
dA,dC,dGTP	300	300	300	300	300	300	300
FITC, fluorescein isothiocyanate							

#### In Situ Hybridization and Detection

##### Centromeric repeats

After PCR amplification, the probes were used without further purification. The DNA solution was diluted 1:5 in 10 mM Tris-HCl/1 mM EDTA. One microliter of each probe was precipitated with 5  $\mu$ g of salmon sperm DNA and 5  $\mu$ g of yeast RNA and resuspended in 10  $\mu$ l of 60% formamide, 2x standard saline citrate (SSC) and 5% dextran sulfate. Probe DNA was denatured at 75°C, 5 min, and immediately applied to the denatured chromosome specimens; a coverslip was added and sealed with rubber cement. The slides were denatured separately in 70% formamide/2x SSC for 2 min at 80°C and dehydrated in an ethanol series. After overnight incubation at 37°C, the coverslips were removed and the slides were washed at 45°C in 50% formamide/2x SSC three times, followed by three washes at 60°C in 0.1x SSC. After a blocking step (in 4x SSC/3% bovine serum albumin for 30 min at 37°C), the

biotinylated probes were detected using streptavidin conjugated to the infrared dye Ultralite 680 (Ultra Diagnostic Corporation, Seattle, WA; final concentration, 2  $\mu\text{g}/\text{ml}$ ); the dig-labeled probes were detected with a rhodamine-labeled anti-dig IgG (Boehringer Mannheim). The fluorescein-1-dUTP (Boehringer Mannheim) labeled probes did not require any immunological detection step. 4',6-Diamidino-2-phenylimdole (DAPI) was used as a chromosome counterstain.

#### Chromosome painting

The amplification products were treated with DNase I to an average size of 150-500 bp. Five microliters of the amplification reaction mixture (50  $\mu\text{l}$ ) was precipitated with 5  $\mu\text{g}$  salmon sperm DNA and 5  $\mu\text{g}$  of yeast RNA, together with 10  $\mu\text{g}$  total human competitor DNA, and then resuspended in 10  $\mu\text{l}$  50% formamide/2x SSC/10% dextran sulfate. The probe was denatured as described above and allowed to preanneal for 1 h at 37°C. Slides were denatured as described for the centromeric repeats. Hybridization took place overnight at 37°C. Slides were washed at 42°C in 50% formamide followed by three washes at 60°C in 0.5x SSC. The biotinylated sequences were detected with streptavidin conjugated to the infrared dye Ultralite 680; the dig-labeled sequences were detected with rhodamine-labeled anti-dig IgG (Boehringer Mannheim). DNP-labeled probes were detected with a monoclonal rat anti-DNP antibody (Novagen) and a secondary goat anti-rat antibody, conjugated to Fluorescein isothiocyanate (Sigma). DAPI was used as a DNA counterstain.

#### Cosmid clones

Eighty ng of each cosmid or phage was precipitated with 20  $\mu\text{g}$  human competitor DNA and 5  $\mu\text{g}$  each of yeast RNA and salmon sperm DNA. The detection of the differently labeled probe DNAs was performed as described above for the chromosome specific libraries.



### Digital Imaging

Images were obtained using a Zeiss Axioskop epifluorescence microscope coupled to a cooled charge coupled device (CCD) camera (Photometrics, Tuscon, AZ, PM512). Camera control and digital image acquisition (8-bit gray scale) employed an Apple Macintosh IIX computer. Fluorophores were selectively imaged using filter cubes specially prepared by Zeiss (Filter 487910 for fluorescein, filter 487915 for rhodamine and filter 487901 for DAPI) to minimize image offsets. The infrared filter (excitation 620-658 nm; dichroic, 650 nm, bandpass, 670-680 nm) is not a precision filter. Images taken using the latter filter were therefore slightly shifted. These were digitally realigned with the probe signals as reference.

Each set of three gray scale fluorescence images revealed probe signals that appeared in only one, in two, or in all three of the images (i.e., the seven combinatorial possibilities). Since the probe-positive regions were visually distinct and were relatively few in number, their combinatorial participation was readily identifiable by visual inspection of the image groups. As a step toward uniquely pseudocoloring these data regions on a combinatorial basis, the regions were isolated and segregated into seven separate (but still spatially aligned) gray scale subimages by using interactive graphics software. Data regions were blended (intensity was averaged) in those cases in which probe signals appeared in more than one of the original images.

The visual identification and manual interactive segregation of data regions was necessary due to limitations of currently available graphics software.

The seven intermediate gray scale images were then separately pseudocolored, a process that converts a gray scale to a tint scale. The pseudocolored images were then recombined

through a simulated overlay. The multicolored composite image was simultaneously merged with a DAPI counterstain image (also pseudocolored) using software developed in our laboratory that combines images by picking maximum signal intensity at each pixel position. The digital imaging technique described above can be implemented on a general purpose computer, e.g., an Apple Macintosh IIX, using known image processing tools, as particularly arranged and operated in accord with the above methodology. A preferred such implementation, termed Gene Join, can be obtained from the Office of Cooperative Research, Yale University, Suite 401, 246 Church St., New Haven, CT 06510.

Photographs were taken with a Agfa matrix procolor slide printer using Kodak 100 HC color slide film.

## RESULTS

### Combinatorial Labeling of Probes by PCR

Chromosome-specific centromeric repeats and chromosome specific DNA libraries are frequently used as probes for fluorescence in situ hybridization (FISH) because of their utility in revealing chromosome aneuploidy or aberrations in interphase cells and tissues as well as the identification of marker chromosomes unrecognizable by conventional banding methods (Tkachuk, D.C. et al. (1991) GATA 8:67-74; Lichter, P. et al. (1991) GATA 8:24-35). Since clones containing such sequences generally have relatively small inserts, ranging in size from a few hundred nucleotides to a few kilobase pairs, we first chose to assess vector PCR as a general method for the combinatorial labeling of such clones. Bio, dig, DNP and fluorescein, all conjugated to dUTP, could be efficiently incorporated during the amplification reaction, alone or in

combination, resulting in selective enrichment of labeled chromosome-specific sequences. Several combinations of nucleotide analogs were tested in order to establish the appropriate concentrations necessary to give an approximately equimolar mixture of each reporter in the probe. These nucleotide concentrations are listed in Table 1 and Table 2. Alphoid DNA clones specific for chromosomes 3, 7, 8, 9, 11, 12 and 18 and chromosome-specific libraries for chromosome 1, 2, 4, 8, 14 and X were then labeled combinatorially by vector PCR. Each combination with Bio, dig and DNP (or fluorescein-dUTP) singularly tested by in situ hybridization and each combination gave comparable signal intensities (data not shown).

#### Simultaneous Detection of Seven Centromere Repeat Probes

The chromosome-specific alphoid DNA clones and the modified nucleotides used to label them are given in Table 5. The biotinylated probes were detected with an infrared fluorophore emitting at 680 nm (Ultralite 680) conjugated to streptavidin. The dig-labeled probes were detected with anti-dig antibodies coupled to rhodamine (630 nm emission) while the probes labeled with fluorescein-11-dUTP (580 nm emission) were detected directly. A separate gray scale image of each fluorophore was then acquired by using the CCD camera system. As shown in Figure 1 (A-C), four pairs of chromosome-specific hybridization signals are seen in each image, as expected from the experimental design. Each of the source gray scale images have been pseudocolored to highlight the hybridization signals. One pair of signals appears uniquely on each of the images (see arrowheads), reflecting those probes that were labeled with only a single reporter. Two other signal pairs appear on two images each, while the third appears on all three images (see arrows). Thus each

probe could be selectively identified by the fluorophore image combination on which the hybridization signal was detected. The gray scale signal regions from the images were segregated, pseudocolored and merged with computer software as described. Figure 1D shows this merged image. Each of the seven centromere probes are seen as distinct colors on the DAPI (blue) counterstained metaphase chromosomes. The probes could also be clearly distinguished after hybridization to fixed human lymphocyte nuclei. Figure 1E shows a merged image of an interphase nucleus hybridized with a mixture of the seven centromere probes.

#### Simultaneous Painting of Six Chromosomes. Detection of a Chromosomal Translocation

Chromosome painting is a powerful and general approach to study chromosomal abnormalities. Here the probes are a complex composite of sequences cloned in plasmid or phage vectors with flow-sorted chromosomes used as the starting DNA source (Pinkel, D. et al. (1988) Proc. Natl. Acad. Sci. USA 85:9139-9142; Cremer, T. et al. (1988) Hum. Genet. 80:235-246). To demonstrate that combinatorial labeling also could be used for whole chromosome analysis, the libraries for chromosomes 1, 2 and 4 were singly labeled with Bio, dig and DNP, while the libraries for chromosomes 8, 14 and X were labeled combinatorially (see Table 6). Each probe set decorated a single chromosome pair when analyzed by FISH, with signal intensities on each fluorophore channel being of similar intensity (data not shown). The merged image (Figure 1F) highlights the six target chromosomes in different pseudocolors while the remaining chromosomes exhibit the blue DAPI counterstain.

Table 6. Labelling of Chromosome-Specific Libraries

	dNTP, $\mu$ M					
	pBS2	pBS14	pBS1	pBS4	pBSX	pBS8
Bio-11-dUTP	75			37.5	37.5	
dig-11-dUTP		75		37.5		37.5
DNP-11-dUTP			37.5		37.5	37.5
TTP	225	225	262.5	225	225	225
dA,dC,dGTP	300	300	300	300	300	300

The PCR-generated libraries can also be used for detection of chromosomal translocations as exemplified in Figure 1G. Metaphase spreads were obtained from lymphocytes of a healthy female donor whose karyotype (Giemsa banding) was shown to be 46,XX,t(2;14)(q37;q22). The metaphase spreads were investigated in order to clarify the origin of an identical translocation detected in the fetus of the donor. Using PCR-generated libraries for chromosome 2 (Bio) and chromosome 14 (dig), the reciprocal character of the translocation could be clearly demonstrated (see arrowheads).

#### Combinatorial Labeling and Gene Mapping

The feasibility of mapping multiple genes simultaneously by using the combinatorial labeling paradigm is demonstrated by the data presented in Figure 1 (H-J). Six different cosmid and phage clones, previously mapped to chromosomes 5, 6, 8, 11, 21 and X in independent experiments, were cohybridized and separate gray scale fluorescence images collected and processed as described above. The merged image on a DAPI-counterstained metaphase spread is shown in Figure 1H. The chromosomal location of each clone, as measured by both fractional length measurements (Lichter, P. *et al.* (1990) *Science* 247:64-69) and *Alu*-PCR hybridization banding (Baldini, A. and Ward, D.C. (1991) *Genomics* 9:770-774), was identical to that obtained before (data not shown). Six cosmid clones with known locations on chromosome 5 were also hybridized simultaneously. Figure 1I shows the distribution of these clones on both

chromosome 5 homologs in a metaphase spread while Figure 1J demonstrates that the relative order of the clones (i.e., the pattern of colors) is maintained in the interphase nuclei of a T lymphocyte. It should be noted that many of the signals appear as doublets, reflecting the fact that these sequences have already undergone DNA replication in this nucleus. Conversion of a probe signal from a singlet to a doublet can be used to monitor the replication timing of DNA segments during S phase.

### DISCUSSION

A procedure that permits the analysis of up to seven probes simultaneously. Combinatorially labeled probes can be produced rapidly and reproducibly by either nick-translation or PCR amplification. However, the latter approach is particularly attractive for labeling clones with relatively small inserts (about 6 kilobases or less) since vector-derived PCR primers permit selective amplification of insert DNA sequences with high efficiency. For example, with the alphoid DNA clones, a typical 50  $\mu$ l amplification reaction mixture yields sufficient labeled probe for about 250 in situ hybridizations. Not surprisingly, the yield for the chromosome library clone pool is lower; nevertheless, 100 ng of template gave enough amplification products to hybridize 10 slides. Reamplification of the primary PCR product pool could also be done without any detectable loss of probe complexity (data not shown). In contrast, using nick-translated plasmid libraries, 200 ng of DNA was required per slide. The negligible amount of labeled vector sequences in the PCR products also reduces the potential for vector sequence cross hybridization, a problem which was described by Nederlof et al. (1990) Cytometry 10:20-27.

The digital imaging capabilities of the cooled CCD camera and the computer software for pseudocoloring and merging signals from combinatorially labeled probes will play an important role in extending the number of simultaneously detectable probes beyond the seven reported here. The CCD camera is sensitive to light over a broad spectrum range. Infrared dyes, such as Ultralite 680, which are not visible by eye, can be imaged quite readily by the CCD camera. A series of fluorophores emitting in the 650 to 900 nm range, have recently been reported (Ernst, L.A. *et al.* (1989) Cytometry 10:3-10); this should increase the number of different fluorophores that can be used combinatorially for probe identification. Furthermore, the infrared dyes, such as Ultralite 680, offer certain advantages over the blue fluorophores, AMCA or Cascade Blue: i) sample autofluorescence is minimal at the longer wavelength, ii) DAPI counterstaining of metaphase chromosomes and interphase nuclei is possible (the emission of DAPI, AMCA and Cascade Blue overlap) and iii) the observed bleed-through of rhodamine signals with the DAPI filter when imaging AMCA fluorescence is more severe than the bleed-through of rhodamine signals using the infrared filter.

Digital imaging of combinatorially labeled probes also circumvents a universally thorny problem in multicolor analysis, that of precise image registration. When filter cubes are moved to collect the fluorescence emission of a single fluorophore, optical imperfections or mechanical motion may cause image displacement relative to each other; these registration offsets can be as large as 1  $\mu\text{m}$ . This is extremely problematic when spatial relationships between signals are critical, such as in gene mapping. However, when multiple probes, combinatorially labeled, are cohybridized, signals from these probes appear on two or more of the separate fluorophore images, thus providing internal reference points

for image registration. Provided that one hybridization signal set is directly tied to the complete image of a metaphase spread or interphase nucleus, i.e., by using a dual bandpass filter (Johnson, C.V. *et al.* (1991) *GATA* 8:75), all images can be aligned, irrespective of the number of separate images to be merged. Fluorescence *in situ* hybridization is becoming an increasingly powerful experimental tool, both for basic research and for clinical applications. The ability to visualize multiple probes simultaneously should streamline the screening of specimens for chromosomal aneuploidies and/or chromosomal rearrangements. This is of particular importance in cases where clinical samples are limited in number. In addition, by incorporating one or more appropriate reference clones (e.g., centromere repeats or unique sequence genes) in the experimental protocol, the assessment of gene dosage (loss of heterozygosity, aneuploidy and mosaics) or defining boundaries of chromosomal deletions should be more definitive and require less statistical analysis. The generation of physical mapping data, using either metaphase or interphase mapping strategies should be facilitated with combinatorial fluorescence as would studies focused on understanding the intranuclear topography of genes and chromosomes. It should be stressed that the assessment of the chromosomal map positions of several combinatorially labeled clones does not necessarily require the pseudocoloring and merging procedures. Displaying the signals separately as gray scale images, as shown in Figure 1 (A-C), allows the physical ordering of probes, since combinatorially labeled clones appear in several gray scale images and can thus be identified. Manual segregation of the images is time-consuming, which in its present format reduces the rate at which clones can be mapped. This limitation can be eliminated by software to automate this step.



The use of commercially available nucleotide analogs conjugated to fluorescein is of particular value for clinical applications since it circumvents time-consuming and sometime troublesome immunological steps required to visualize haptenized probes. In addition, this results in an improved signal/noise ratio, which could enhance overall detection sensitivity, especially if a cooled CCD camera were used for imaging. It can be expected that other nucleotides with additional conjugated fluorophores will be available soon, which would both simplify and expand the combinatorial labeling strategy for multicolor hybridization assays even more.---

In the Claims

Cancel claims 1-3, 5-7, 9-10 and 12-16.

Add the following new claims 19-35:

19. A method of DNA hybridization for detecting target chromosomal DNA in situ in interphase cells, comprising:
  - a) providing labeled probe DNA having sequences specifically hybridizable to target chromosomal DNA, but having repetitive sequences which cross-hybridize to non-target chromosomal DNA, and competitor DNA containing the repetitive sequence, the labeled probe DNA and the competitor DNA comprising fragments less than about 500 nucleotides in length;
  - b) combining
    - i) the labeled probe DNA;
    - ii) the competitor DNA; and

iii) a sample of interphase cells, or nuclei thereof, treated to render chromosomal DNA therein available for hybridization with the probe DNA, under hybridization conditions wherein cross-hybridization between repetitive sequences in the probe DNA and the sample is sufficiently suppressed to allow the probe DNA to hybridize essentially specifically to the target chromosomal DNA; and

c) detecting the labeled probe DNA in order to detect the target chromosomal DNA.

20. The method of claim 19, wherein the labeled probe DNA and the competitor DNA are combined before combination with the sample of interphase cells or nuclei thereof.
21. The method of claim 20, wherein the labeled probe DNA and the competitor DNA are combined under conditions which allow the repetitive sequences in the competitor DNA and repetitive sequences in the labeled probe DNA to preanneal and to thereby suppress cross-hybridizing repetitive sequences sufficiently to produce labeled probe DNA hybridizable essentially specifically to the target chromosomal DNA.
22. The method of claim 19, wherein the interphase cells are tumor cells or nuclei thereof.
23. The method of claim 19, wherein the interphase cells comprise uncultured cells from amniotic fluid, or nuclei thereof.
24. The method of claim 19, wherein the target chromosomal DNA is an individual chromosome or a region thereof.

25. The method of claim 24, wherein the labeled probe DNA is DNA obtained from a library of DNA of the target chromosome.
26. The method of claim 19, wherein the labeled probe DNA and the competitor DNA comprise DNA fragments ranging from 150-250 nucleotides in length.
27. The method of claim 19, wherein the competitor DNA for suppression of repetitive sequences is total human genomic DNA.
28. The method of claim 19, wherein a carrier DNA is added to the combination of labeled probe DNA, competitor DNA and the sample preparation.
29. A method of claim 22, for simultaneous detection of more than one chromosome, or region thereof, wherein probe DNA for each chromosome, or region thereof, is labeled with a different fluorophor or a different combination of fluorophors to yield an optically distinguishable signal.

30. A method of in situ hybridization for distinguishably labeling individual human chromosomes, or regions thereof, comprising
- a) providing labeled probe DNA for each chromosome, or region thereof, to be visualized, each probe DNA having sequences specifically hybridizable to target chromosomal DNA, but having repetitive sequences which cross-hybridize to non-target chromosomal DNA and each probe DNA being labeled with a different fluorophor or a different combination of fluorophors to generate optically distinguishable signals; and
  - b) combining
    - i) the labeled probe DNA for each chromosome, or region thereof;
    - ii) a competitor DNA containing the repetitive sequences; and
    - iii) a sample of cells, or nuclei thereof, treated to render chromosomal DNA therein available for hybridization with the labeled probe DNA, under hybridization conditions wherein cross-hybridization between repetitive sequences in the probe DNA and the sample is sufficiently suppressed to allow each probe DNA to hybridize essentially specifically to the target chromosomal DNA, to thereby distinguishably label individual chromosomes.

31. A method of in situ hybridization for visualizing an individual human chromosome, or region thereof, comprising
- a) providing biotinylated DNA fragments smaller than 500 nucleotides derived from a DNA library of a target chromosome, or region thereof, for visualization;
  - b) combining the biotin-labeled DNA fragments with fragments of total genomic DNA having the same size distribution as the labeled fragments and incubating the mixture under initial conditions that allow denaturation of the fragments and, subsequently, under conditions that promote annealing of fragments containing repetitive sequences but not fragments containing chromosome-specific sequences, to produce probe DNA essentially specific for the target chromosome;
  - c) combining the probe DNA with a cellular preparation, treated to render target chromosomal DNA available for hybridization with the probe DNA, under conditions which allow the probe DNA to hybridize to the target chromosomal DNA; and
  - d) detecting the probe DNA by incubating the preparation with an avidin conjugated fluorophor and the detection of the fluorescent signal.

32. A method of in situ hybridization for simultaneously visualizing individual human chromosomes, or regions thereof, comprising
- a) providing labeled probe DNA for each chromosome, or region thereof, to be visualized, each probe DNA having sequences specifically hybridizable to target chromosomal DNA, but having repetitive sequences which cross-hybridize to non-target chromosomal DNA and each probe DNA being labeled with a different fluorophor or a different combination of fluorophors to generate optically distinguishable signals;
  - b) combining
    - i) the labeled probe DNA for each chromosome, or region thereof;
    - ii) a competitor DNA containing the repetitive sequences; and
    - iii) a sample of cells, or nuclei thereof, treated to render chromosomal DNA therein available for hybridization with the labeled probe DNA, under hybridization conditions wherein cross-hybridization between repetitive sequences in the probe DNA and the sample is sufficiently suppressed to allow each probe DNA to hybridize essentially specifically to the target chromosomal DNA; and
  - c) detecting the optically distinguishable signals generated by each probe DNA to visualize each chromosome.

33. A method according to claim 32, wherein the detecting step includes the steps of
  - a) generating one or more digital images of the hybridized chromosomal DNA; and
  - b) visually emphasizing those portions of the digital images which represent optically distinguishable signals associated with a fluorophor or combination thereof.
34. A method according to claim 33, wherein the generating step includes the step of producing each the digital image by imaging the hybridized chromosomal DNA with a filter having a bandpass corresponding to each of a respective one of the fluorophors.
35. A method according to claim 34, wherein the visually emphasizing step includes the steps generating an image of the hybridized chromosomal DNA by
  - a) assigning a respective color to each fluorophor; and
  - b) substituting, for those portions of the digital images which represent optically distinguishable signals associated with the fluorophors, or combinations thereof, respective colors assigned thereto.

Remarks

This amendment is submitted as part of a file-wrapper-continuation in part of Application Serial No., 07/271,609 filed November 15, 1988. Figure 15 and a new Example III have been added. All claims have been replaced by newly written claims 19-35.

The new claims define the invention more particularly and comply with the requirements of §112. Support for the new claims can be found throughout the specification and in the claims as originally filed. Specifically, support for claim 19 appears, inter alia, at pages 14-19 and at pages 39-52. Sources of the labeled probe DNA are described at page 15, lines 3-28. The labeling of the DNA is described at page 15, line 29 and page 16, line 12. The preannealing step of claims 21 and 30 is described at page 17, line 27 - page 18, line 26 and in further detail at pages 35-36 and at pages 39-52. Application of the method to interphase cells such as tumor cells (claim 22) and uncultured interphase cells from amniotic fluid (claim 23) is described, inter alia, at page 20, lines 3-9 and page 29, line 15. Support for the fragment size distributions recited in claims 19, 26 and 30 is found at page 16, lines 15-18. Support for the use of total genomic DNA as competitor DNA (claim 27) is found at page 17, lines 7-11. Carrier DNA (claim 28) is described at page 18, lines 14-22. Combinational labeling for detection of more than one chromosome (claims 29-34) is described at pages 23-25 and in the material added by amendment.

Applicants' invention is a method of in situ hybridization for detecting chromosomal DNA in interphase nuclei. The method can be used to label entire individual human chromosomes, or regions thereof, or to visualize a plurality of different chromosomes simultaneously in order to evaluate numerical and structural aberrations of chromosomes (for example, chromosomal



aneuploidy and chromosomal deletions, rearrangements and translocations). In Applicants' method, the hybridization probe can be made from libraries of DNA from individual chromosomes. Such libraries are readily available for all human chromosomes. The DNA from these libraries contains sequences specific to a chromosome but it also contains sequences which are non-specific such as the ubiquitous repetitive sequences Alu and KpnI which appear in all chromosomal DNA. Thus, although this DNA will hybridize to the target chromosome, it will also cross-hybridize to other chromosomes, prohibiting specific detection of the target chromosome.

According to the method of this invention, probe DNA fragments of less than 500 nucleotides are used. The cross-hybridization of probe repetitive sequences to cognate repetitive sequences in non-targeted chromosomes can be suppressed by a competitor DNA containing the repetitive sequences. For example, DNA derived from a single chromosome library can be suppressed with total human genomic DNA to provide chromosome-specific hybridization. In one embodiment, the probe DNA and competitor DNA are preannealed to suppress cross-hybridizing sequences. The probe DNA is then hybridized to cellular or chromosomal preparation to specifically label a target chromosome, or a region of the chromosome. The label associated with the probe is detected to visualize the individual chromosome, or region thereof.

By combinational labeling, probe sets specific for different chromosomes can be distinguished, allowing for simultaneous visualization of more than one chromosome in a sample. Probe can be labeled different fluorophors and different combinations of fluorophors to generate optically distinguishable signals for visualization of individual chromosomes.

This method of in situ suppression hybridization permits rapid analysis of samples and decreases the time to make diagnosis of genetic disorders such as Down's Syndrome. The procedure can be without the need to culture cells (e.g., fetal cells obtained by amniocentesis) or to obtain metaphase spreads (e.g., interphase tumor cells).

The Examiner objected to the specification and rejected all claims under §112, first paragraph. This objection is obviated by amendment of the claims. As pointed out above, the new claims are fully supported by the specification.

All claims were rejected under §103 as being unpatentable over SU 1203108 in view of Singer et al. and Dattagupta et al. This rejection is obviated by amendment of the claims. SU 1203108 teaches a method detecting human chromosome 11 using a probe specific for the pre-central region of chromosome 11. This is an example of one probe which is specific for a particular chromosome. In Applicants' method, probes are fragments less than 500 nucleotides which contain repeat and specific sequences and they are made specific by suppressing non-specific sequences. Such probes can be made for any chromosome from, for example, libraries of genomic chromosomal DNA. Singer describes a method of in situ hybridization and Dattagupta teaches a method for labeling nucleic acids. None of these references teaches or suggests suppression of repetitive sequences in a probe DNA by a competitor DNA for specific hybridization in interphase cells. Further, these references do not teach or suggest combinational labeling techniques for visualizing more than one chromosome simultaneously by in situ hybridization.

All claims were rejected under §102(a) as being clearly anticipated by Cremer et al. and [sic; or?] Pinkel et al. This rejection is obviated by amendment of the claims.

Cremer et al. teach that two repetitive DNA sequences which are confined to chromosomes 1 and 18 can be detected with labeled hybridization probes. Thus, the probes can be used to detect these particular chromosomes. Some cross-hybridization of the probe for chromosome 18 (probe 18c) was observed. There is no teaching of the claimed hybridization assay for specifically detecting chromosomes in interphase cells or the labeling technique to visualize a plurality of chromosomes.

Pinkel et al. teach a method of staining human chromosomes in interphase nuclei by in situ hybridization. According to their method, "labeled probe DNA is mixed with unlabeled human genomic DNA, denatured and incubated before applying it to the denatured target cells". The authors do not teach or suggest suppression of repetitive sequences in the probe DNA. As in Cremer et al., there is no teaching or suggestion of the claimed method of combinatorial labeling for visualizing more than one chromosome simultaneously in a sample.

All claims were rejected under §102(b) as being clearly anticipated by Gray et al. and [sic; or ?] Landegent et al. This rejection is obviated by amendment of the claims.

Gray et al. teach fluorescence hybridization in metaphase spreads to human chromosome 21 using a composite collection of biotin-labeled probes from a library made from DNA isolated from human 21 chromosomes. In one approach to eliminate non-specific hybridization, unlabeled whole genomic human DNA was used for prehybridization and during hybridization to block binding of non-specific repeat sequences in the biotin-labeled probe DNA. Applicants' method can be used to label chromosomes for interphase cells. Further, Applicants' method of combinatorial labeling can be used to visualize more than one chromosome in the same sample.

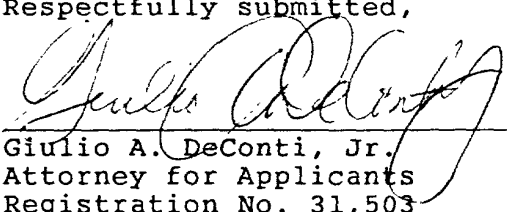
Landegent et al. describe an in situ hybridization technique for chromosomal localization in which the presence of highly repetitive sequences is eliminated with competition hybridization with Cot-1 DNA. As in Gray et al., the work described was performed in metaphase spreads. There is no teaching or suggestion of whether or how in situ hybridization can be performed in interphase cells to result in chromosome specific labeling. Further, there is no teaching or suggestion of the claimed method of combinatorial labeling for visualizing more than one chromosome simultaneously in a sample.

All claims were rejected under §102(f). The basis for this rejection is uncertain because the Examiner has not cited a reference or provided other evidence supporting the contention that the claimed invention was not made by Applicants. The Examiner states that the rejection is based on the Cremer et al. reference. If the Examiner means the same Cremer et al. reference cited in support of the rejection under §102(a), then Applicants' comments directed to that rejection apply to the rejection under 102(f) and it should be withdrawn.

#### Conclusion

The claims are now in condition for allowance and Applicants respectfully request early indication of such. If the Examiner feels that a telephone conversation will expedite prosecution of the application, she is invited to call the undersigned at (617) 227-7400.

Respectfully submitted,

  
Giulio A. DeConti, Jr.  
Attorney for Applicants  
Registration No. 31,503

Dated: February 14, 1992

LAHIVE & COCKFIELD  
60 State Street  
Boston, MA 02109  
(617) 227-7400

-1-

DELINEATION OF INDIVIDUAL HUMAN CHROMOSOMES  
IN METAPHASE AND INTERPHASE CELLS BY IN SITU  
SUPPRESSION HYBRIDIZATION

Description

Background

Chromosome banding techniques have facilitated the identification of specific human chromosomes and presently provide the major basis upon which chromosomal aberrations are diagnosed. The interpretation of chromosome banding patterns requires skilled personnel and is often technically difficult, especially with respect to detecting minor structural changes and when analyzing complex karyotypes, such as those of highly aneuploid tumor cells. An additional complexity is that readable metaphase chromosome spreads are sometimes very difficult or impossible to prepare from certain cell types or tissues. Alternative methods for identifying chromosomal aberrations would be valuable because they could augment current methods of cytogenic analysis, particularly if such alternative methods were applicable to both mitotic and interphase cell populations.

Over the past few years, a considerable body of evidence has been obtained which indicates that the DNA of individual chromosomes occupy focal territories, or spatially cohesive domains, within mammalian interphase nuclei. Cremer, T. et al., Hum. Genet., 60:46-56 (1982); Hens, L. et al., Exp.

Cell Res., 149:257-269 (1983); Schardin, M. et al., Hum. Genet., 71:281-287 (1985); Manuelidis, L., Hum Genet., 71:288-293 (1985); and Pinkel, D. et al., Proc. Natl. Acad. Sci. USA, 83:2934-2938 (1986). These observations suggest that chromosome-specific probe sets could be used to detect numerical or structural aberrations of chromosomal domains in non-mitotic cells, an approach termed "interphase cytogenetics". Cremer, T. et al., Hum. Genet., 74:346-352 (1986). Indeed, recent in situ hybridization studies have demonstrated the prenatal diagnosis of trisomy-18 with interphase cells and the detection of numerical chromosomal abnormalities in tumor cells lines using chromosome-specific repetitive DNAs as probes. Cremer, T. et al., Hum. Genet., 74:346-352 (1986) and Cremer, T. et al., Exp. Cell Res., 176:119-220 (1988). All chromosome-specific repetitive DNAs reported to date are localized to discrete subregions of each chromosome and, thus, such DNA probes are unsuitable for analyses of many types of chromosomal aberrations (e.g., translocations and deletions). If it were possible to detect uniquely the spectrum of sequences comprising a specific chromosome, analysis of aberrations of chromosomal domains in non-mitotic cells would be possible. Furthermore, such a general labeling technique would make it possible to address fundamental questions concerning the spatial organization of chromosomal DNA within interphase nuclei.

Disclosure of the Invention

The subject invention relates to a method of detecting, identifying and/or quantitating selected individual chromosomes in mammalian mitotic or interphase cells, by means of chromosomal in situ suppression (CISS) hybridization and its use in analyzing cells for the occurrence of chromosomes, chromosome fragments, or chromosome aberrations, such as those associated with a condition or disease. In the method of the present invention, chromosome-specific probes (DNA or RNA) are combined with a sample to be analyzed, in such a manner that an individual chromosome(s) of interest is labeled and the complex spectrum of sequences which comprise the chromosome can be detected. The probes used in the present method are of high genetic complexity and can be appropriately-selected cloned DNA or RNA fragments, used individually or in pools, or chromosome library DNA.

The method of the present invention, referred to as CISS hybridization, is particularly useful because it can be used to specifically stain individual mammalian chromosomes at any point in the cell cycle. It can be used to assess chromosomal content, particularly chromosome aberrations (e.g., deletions, rearrangements, change in chromosome number) which, until the present invention, it has been time-consuming and/or difficult, if not impossible, to detect. The method is useful in providing a rapid and highly specific assessment of individual mammalian chromosomes in any context

(e.g., diagnosis and/or monitoring of a genetic condition or a disease state) in which such an assessment is desired.

#### Brief Description of the Drawings

Figure 1 presents an outline of the CISS hybridization method of the present invention.

Figure 2 shows suppression from cross-reacting sequences within a chromosome 7-derived DNA library by different concentrations of human competitor DNA. Biotin-labeled chromosome 7 DNA inserts (20  $\mu\text{g/ml}$ ) were prehybridized for 20 minutes with human genomic DNA prior to hybridization with metaphase chromosome spreads and detection with FITC-labeled avidin. Human DNA concentrations were: A, 0  $\mu\text{g/ml}$ ; B, 50  $\mu\text{g/ml}$ ; C, 100  $\mu\text{g/ml}$ ; D, 200  $\mu\text{g/ml}$ ; E, 1000  $\mu\text{g/ml}$ ; F, same metaphase spread as in E post-stained with DAPI. Genomic salmon DNA was added to each sample to adjust the final DNA concentration to 1.0 mg/ml (see the text for details). The arrows mark the target chromosome 7 and the arrowheads mark additional strong signals on non-7 chromosomes. All negatives printed were exposed and developed under identical photographic conditions.

Figure 3 shows the effect of pre-annealing time on the specificity and strength of the hybridization signal. Biotin-labeled chromosome 7 DNA inserts (20  $\mu\text{g/ml}$ ) were preannealed with 200  $\mu\text{g/ml}$  human competitor DNA for the following times, prior to hybridization to metaphase A, 0 minutes; B, 2 minutes; C, 5 minutes; D, 20 minutes.



Figure 4 shows decoration of A chromosome 1, B chromosome 7, C chromosome 4, D chromosome 18, E chromosome 13 and F chromosome 20 in normal human lymphocytes. Only the chromosome 13 insert DNA pool shows significant cross-hybridization to other chromosomes after the prehybridization suppression step. The detection of chromosome 20 (F) was done with the entire chromosome library (including  $\lambda$  phage arms) and detected with avidin-alkaline phosphatase using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) as the enzyme substrate mixture. The signal of chromosome 1 (A) was amplified by the sandwich technique of Pinkel et al. (1986).

Figure 5 shows chromosome domains in human lymphocyte nuclei delineated by preannealed chromosome library DNA inserts. Hybridization to acetic acid-methanol fixed nuclei was detected by fluorescein isothiocyanate (FITC)-conjugated avidin (A-E) or alkaline phosphatase-conjugated avidin (F). Domains are shown for chromosome 1 (A,B), chromosome 7 (C,D) and chromosome 18 (E,F). A predominant staining of the centromere region is seen within the chromosome 7 domains, reflecting preferential hybridization of the chromosome 7-specific alphoid DNA repeat; a similar signal distribution on metaphase chromosomes was also observed in this particulate experiment.

Figure 6 shows chromosomal in situ suppression (CISS) hybridization of chromosome 1 inserts to metaphase spreads of glioma cell lines detected with

avidin-FITC (A,C) and poststained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (B,D). A,B TC 620 show two apparently complete 1 chromosomes (small arrows in B) and two marker translocation chromosomes (arrowheads) specifically decorated by these inserts (A). One of the two marker chromosomes contains a lp (lower left), the other a lq arm (lower right); the lp terminal (relatively GC rich region) in the two normal chromosomes and submetacentric marker is less completely delineated. Also, the lql2 regions here show little decoration in contrast to most experiments. X 950. C,D Typical TC 593 metaphase spreads show six specifically decorated chromosomes. Three acrocentric marker chromosomes all with truncation of lp show particularly intense fluorescence of repeats that localize to lql2 (arrows in C). In two of these, lq arms appear to be complete, while a major deletion is obvious in the third (the arrow in D). A fourth decorated chromosome (small arrowhead in C,D) again shows a major deletion of the distal part of lq, but has retained an apparently complete lp arm. A fifth submetacentric chromosome (large arrowhead) contains an apparently complete lp arm; the DNA of its short arm has not been identified. Note the similarity of this marker to one of the marker chromosomes of TC 620 (lp) described above. The sixth entirely decorated chromosome is an iso (lp) as demonstrated by DAPI-banding (open arrows). X 1200

Figure 7 shows CISS hybridization of chromosome 4 library inserts detected with a avidin-FITC; interphase nuclei of TC 593 (A-C) and TC 620 (D). Note that the two apparently complete interphase domains are close to each other in B, but widely separated in A and C. TC 620 interphase nucleus (D) shows four chromosome 4 interphase domains of largely different sizes. Metaphase spread of TC 593 (E) shows two apparently complete 4 chromosomes, and a small decorated region (arrow) in a submetacentric chromosome. This marker with translocated 4 sequences was observed in about 30% of the spreads. TC 620 metaphase spread (F) shows one apparently complete chromosome 4 and three translocation markers (t) containing different amounts of chromosome 4 material. G-J Double hybridization of biotinylated chromosome 7 inserts and an aminoacetylfluorene (AAF)-modified 7-specific alphoid repeat. G Chromosome 7 inserts depict five entirely decorated metaphase chromosomes. Four of them are complete 7 chromosomes, the fifth (arrow) is an iso (7p) (see Fig. 3E). H The same field as G showing AAF-7 alphoid signals on only four decorated chromosomes; no signal is detected on the iso (7p). I An interphase nucleus of TC 593 shows five domains delineated by chromosome 7 inserts; four of these are labeled by 7 alphoid probes (J). The arrow in I represents the iso (7p) marker in interphase.

Figure 8 shows CISS hybridization of library inserts of chromosomes 7 and 18 to metaphase spreads of glioma cell lines detected with avidin-FITC. X

875. A,B TC 620 hybridized to chromosome 7 inserts. Three apparently normal 7 chromosomes and an additional translocation chromosome containing 7 sequences are indicated by large arrowhead in A; DAPI-stained complete chromosomes are indicated by the small arrows in B. Other studies (see the text) indicated a translocation of 7pter-q11 in the marker chromosome (large arrowhead). D, The corresponding frame counterstained with DAPI. E, Metaphase spread from pseudotetraploid TC 593 shows five chromosomes highlighted by 7 library inserts. The metacentric chromosome (m) represents the iso (7p) marker typical for this line (see also Fig. 2G). Insert chromosomes (small arrows) show DAPI-stained normal and metacentric 7 chromosomes. The landmark band 7q21 and a block of constitutive heterochromatin at 7q11 are both prominent on the normal chromosome 7 insert, (arrows) but not present on the marker chromosome. Instead both arms of the latter show a mirror-like staining pattern with a faint distal band at 7p21. F,G TC 593 hybridized to chromosome 18 inserts. Four decorated 18 chromosomes are shown in F and three of them in G by DAPI staining are clearly translocated (t).

Figure 9 is a summary chromosome idiogram of complete and aberrant chromosomes detected by CISS hybridization of library inserts of chromosomes 1, 4, 7, 18 and 20 in glioma cell lines TC 620 (left) and TC 593 (right). G-bands (black) are shown with approximate breakpoints suggested by our data; the shaded areas with a wavy pattern are from other

chromosomes that constitute part of the marker translocation chromosomes. The black dot beside two of the TC 620 translocated 4 segments indicates that the assignment of the chromosome 4 material is based on circumstantial evidence (e.g., size measurements). A small translocation of chromosome 18 material in ca. 20% of TC 593 metaphase spreads (+) also could not be further identified. Note the over-representation of 7p in both cell lines.

Figure 10 shows representative prophase (A) and interphase nuclei (B-F) reflect metaphase abnormalities in glioma lines (cf. Figs. 1-3). A, Detection of the 7p translocation (t) in a prophase TC 620 nucleus. X 1,000. B, Five well-separate chromosome 7 domains detected in TC 593. X 1,240. C, Two large and one very small 18 domains (indicated by arrow head) in TC 620. X 1,450. D, Four chromosome 18 domains detected in TC 593; one of these signals (arrow) appears smaller. X 1,450. E, Four chromosomal 1 domains in TC 620 (cf. Fig. 1A, B). X 1,200. F, At least five chromosome 1 domains in TC 593; one (arrow head) is appreciably smaller than the others. X 1,250. G,H, CISS hybridization of a technically poor metaphase spread of TC 593 poststained with DAPI (G) still highlights four distinct chromosomes bearing 18 sequences (H). X 1,000.

Figure 11 is a graphic representation of the interphase and/or metaphase counts of chromosomes 7 (A-C), 22 (D) and 4 (E) by CISS hybridization. Interphase counts were performed on 150 nuclei of

well-hybridized preparations. For metaphase counts > 25 complete DAPI-stained spreads were evaluated. A-C Counts of 7 specific alphoid repeats (white columns) compared to 7 library inserts (shaded columns): A, interphase nuclei of phytohemagglutinin-stimulated human lymphocytes (46,XY); B, TC 620 interphase nuclei (7-specific alphoid repeat) and metaphase spreads (7 library inserts); C, TC 593 interphase nuclei (7-specific alphoid repeat) and metaphase spreads (7 library inserts). High stringency hybridization (see Materials and methods) of 7 alphoid repeat was used to avoid cross-hybridization to other chromosomes. In cases of double hybridization with both 7 library inserts and alphoid repeat (shown in Fig. 2 G-I) standard conditions with 50% formamide were sufficient to avoid cross-hybridization, possibly due to the presence of human competitor DNA. D, Counts of chromosome 22 (library inserts) in metaphase spreads of TC 620 (black columns) and TC 593 (shaded columns). For comparison, CISS hybridization was simultaneously performed with 7 library inserts in these experiments as an internal control (see C,D and Fig. 7). E, Interphase counts (white columns) and metaphase counts (shaded columns) compared in TC 593 hybridized with chromosome 4 inserts. Note the ratio of signal preparations 2:3 are the same in metaphase and interphase.

Figure 12 shows a TC 620 metaphase spread after double hybridization with inserts from chromosomes 7

and 22 (both labeled with biotin and detected with avidin-FITC). Two strongly decorated 22 chromosomes (arrows), three complete 7 chromosomes, and the metacentric marker chromosome containing 7pter-q11 are also seen.

Figure 13 is a graphic representation of the relative size of decorated normal and aberrant chromosomes 4, 7 and 18 in typical metaphase spreads (n=24) from glioma cell lines TC 593 and TC 620. Individual areas were normalized so that a complete chromosome is represented by an area of 1 (see legend to Table 1). The total added signals reflect the number of specific chromosome equivalents present. The white regions correspond to apparently normal chromosomes, the black regions indicate small free chromosome segments entirely decorated by specific library inserts, and translocated segments are shaded. One of the three translocated 18 chromosomes in TC 593 represents a complete chromosome by this measurement (indicated by the black dot), while the two other translocations are slightly smaller, possibly due to the small sample size.

Figure 14 shows specific labeling of human chromosome 21 by CISS hybridization with biotinylated DNA probe sets. A, plasmid pPW519-1R (6-Kb insert) hybridized to a normal lymphocyte metaphase spread. Signals are located on the termini of 21q (see 4',6-diamidino-2-phenylindole (DAPI)-stained chromosomes in Inset) as verified by DAPI banding (not shown). B and C, Normal human

lymphocyte metaphase (B) and nuclei (C) after hybridization with the 94 Kb plasmid pool probe set. The terminal band 21q22.3 is specifically labeled. D and E, Signals on trisomy 21 (47, + 21) lymphocyte metaphase spreads after hybridization with the 94 Kb probe set (D) or chromosome 21 library DNA inserts with the CISS hybridization (14) protocol. (E) Three chromosomes 21 are entirely delineated by the library inserts; additional minor signals (see text) are indicated by arrowheads (also in G). (F-J) Labeling of trisomy 21 lymphocyte nuclei by the library inserts (F and G; compare with E).

#### Detailed Description of the Invention

The present invention is based on a hybridization strategy in which suppression of hybridization signals from ubiquitous repeated DNA sequences is achieved by using total DNA in a reannealing procedure which is based on rapid reassociation kinetics. The hybridization method of the present invention referred to as chromosomal in situ suppression (CISS) hybridization because of the selective suppression of such signals, has been shown to result in specific cyto-staining of one or more selected individual chromosomes, particularly human chromosomes, at any point in the cell cycle and has been used to detect, identify and quantitate chromosomal aberrations in both mitotic cells and interphase cells (i.e., interphase nuclei).

Described below and in greater detail in the Examples, are the following:



1. specific staining in mitotic and interphase cells of individual human chromosomes, by the method of the present invention (CISS hybridization), using chromosome-specific probe sets which are of high genetic complexity (i.e., chromosome library DNA, cloned DNA fragments);
2. specific staining of metaphase and interphase tumor cells by CISS, using chromosome-specific library probes; and
3. rapid detection in mitotic and interphase cells from a variety of sources of aberrations in a human chromosome (chromosome 21) which associated with a genetic condition (Down syndrome), using CISS hybridization.
4. demonstration that a nested set of chromosome specific unique sequence probes used to identify chromosome aberrations and to detect genetic disease (e.g., Down Syndrome).

#### Specific Staining of Individual Human Chromosomes

By use of the CISS hybridization method, individual (such as the X and Y chromosomes or homolog pairs of chromosomes 1-22) human chromosomes have been specifically stained in both mitotic and interphase cells. This has been carried out in both metaphase spreads and interphase nuclei and has been used to stain or label one selected (individual) chromosome and to stain or label multiple selected (individual) chromosomes simultaneously, using, respectively, signal-probe CISS hybridization and

multi-probe CISS hybridization in conjunction with an appropriate detection method. The method is represented schematically in Figure 1.

Specific Chromosome Staining Using Genomic DNA Libraries and Cloned DNA

CISS hybridization was carried out as follows, to produce specific staining of individual human chromosomes, using commercially-available genomic DNA libraries that originated from flow-cytometry sorted human chromosomes and cloned DNA fragments. Van Dilla, M.A. et al., Biotechnology, 4:537-552 (1986). Suppression of hybridization signals from ubiquitous repeated sequences, such as the Alu and KpnI elements, was achieved using total human DNA in a reannealing procedure that is based on rapid reassociation kinetics. Similar principles have been used by others to facilitate the selective hybridization of unique sequence subsets from cosmid DNA clones for Southern blotting and in situ hybridization experiments. Sealey, P.G. et al., Nucleic Acids, 13:1905-1922 (1985); and Landegent, J.E. et al., Hum. Genet., 77:366-370 (1987). Specific labeling of individual chromosomes in both metaphase spreads and interphase nuclei, is carried out (and shown to have occurred) in the following manner, which is described in detail in the Examples. The feasibility of using computer-assisted optical sectioning for 3-D reconstruction of chromosomal domains for the

analysis of nuclear topography was also demonstrated in conjunction with CISS hybridization.

Initially, genomic DNA from a selected chromosome or selected chromosomes is prepared for use as probe DNA. Genomic DNA is available from several sources. For example, one or more genomic DNA libraries, each containing the chromosome of interest (a chromosome-derived library), is used to produce the necessary DNA probes. Such libraries can be commercially-available genomic DNA libraries that originated from flow-cytometry sorted human chromosomes. These are available from the American Type Culture Collection (Rockville, MD). Such DNA libraries for human chromosomes 1, 4, 7, 8, 9, 12, 13, 14, 16, 17, 18, 20, 21, 22 and chromosome X have been used in the present method, as described in the Examples. Other commercially available genomic DNA libraries or genomic DNA libraries from noncommercial sources can also be used. Alternatively, individual plasmid, phage, yeast artificial chromosomes with non-yeast DNA inserts, and cosmid DNA clones can be used as a source of DNA probes for a selected individual chromosome or multiple selected chromosomes. In the case of DNA from a genomic library, the DNA can be separated as a pool from the vector containing it, prior to labeling with a detectable signal, or can be used without separation from the vector.

Probes are labeled with a detectable signal, which can be a fluorescent reporter, one member of a specific binding pair (e.g., biotin-avidin or

ligand-antibody), or an enzyme. DNA removed from the vector is labeled by nick translation (using, for example, Bio-11-dUTP), by random primer extension with (e.g., 3' end tailing), for example, the Amersham multiprime DNA labeling system, substituting dTTP with Bio-11-dUTP, or other appropriate technique. In the case of DNA which has not been separated from the vector, biotin labeling is carried out directly by nick translation, using standard techniques. Brigati et al., Virology, 126:32-50 (1983). Other labels can be added in a similar manner (e.g., 2,4-dinitro phenol, digoxin).

Probe size is carefully selected and controlled in order to facilitate probe penetration and to optimize reannealing hybridization. In general, labeled DNA fragments smaller than 500 nucleotides are used, and, more generally, the majority of the probes are 150-250 nucleotides in length. Probes of this length are made from longer nucleotide sequences using publicly available restriction enzymes or known techniques for producing and recovering appropriately-sized fragments. It is also possible, if the nucleotide sequence of a selected chromosome is known, to synthesize an oligonucleotide having that sequence, using known techniques. Such oligonucleotides, once labeled, can be used to decorate specific chromosomal regions. For example, oligonucleotide probes which specifically hybridize to telomeric sequences of mammalian chromosomes have been identified. Moyuif

et al., Proceedings of the National Academy of Sciences, USA, Sept. 1988.

Competitor DNA, which is DNA which acts to suppress hybridization signals from ubiquitous repeated sequences, will be selected as needed (e.g., based on the mammal whose chromosomes are being analyzed). In the case of analysis of human chromosomes, competitor DNA is total human DNA which acts to suppress hybridization from ubiquitous repeated sequences, such as the Alu and the KpnI elements. It is available from many sources. For example, human genomic DNA from placenta or white blood cells can be prepared using known techniques, such as that described by Davis et al. Davis, L.G. et al., Basic methods in molecular biology, Elsevier, N.Y./ Amsterdam (1986). It is digested, using standard methods (e.g., with DNase), to produce competitor DNA fragments within the same size distribution as the probe DNA.

DNA from another source, which will compete with only a small portion of the human DNA and which is used, as necessary, to adjust the total (final) DNA concentration of the hybridization mixture will also be included, as needed. This DNA is referred to as carrier DNA. This DNA is produced or treated, using standard methods, so that it is within the same size distribution as the probe DNA.

Initially, probe DNA bearing a detectable label and competitor DNA are combined under conditions appropriate for preannealing to occur. The quantity of probe DNA combined with competitor DNA is

adjusted to reflect the relative DNA content of the chromosome target. For example, chromosome 1 contains approximately 5.3 times as much DNA as is present in chromosome 21. Probe concentrations were 30  $\mu\text{g/ml}$  and 5  $\mu\text{g/ml}$ , respectively. When total genomic library DNA is used as the probe mixture (instead of purified DNA inserts), approximately 10 times as much labeled DNA is added to compensate for the vector sequences, which are present in large quantities. Only twice as much labeled library DNA is added in the case of the libraries LAOXNL01 (X chromosome) and LA16NL02 (chromosome 16) because the human DNA inserts constitute almost half of the total library DNA. Carrier DNA, such as trout or salmon testis DNA, is added to bring the total DNA concentration to a predetermined level, if necessary. As described herein, sufficient salmon testis DNA was added to result in a final DNA concentration of 1.0 mg/ml in the hybridization mixture (which includes all three types of DNA: probe DNA, competitor DNA and DNA which does not significantly compete).

The resulting hybridization mixture is treated (e.g., by heating) to denature the DNA present and incubated at approximately 37°C for sufficient time to promote partial reannealing.

The sample containing chromosome DNA to be identified (specifically labeled) is also treated to render DNA present in it available for hybridization with complementary sequences, such as by heating to denature the DNA. The hybridization mixture and the

sample are combined, under conditions and for sufficient time conducive to hybridization. After sufficient time, detection of specific labeling of the chromosome target is carried out, using standard techniques. For example, as described in the Examples, a biotinylated probe is detected using fluorescein-labeled avidin or avidin-alkaline phosphatase complexes. For fluorochrome detection, samples are incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin DCS (see Example 1). Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat anti-avidin D antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples are incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (e.g., in AP-buffer, as described in Example 1). The enzyme reaction is carried out in, for example, AP buffer containing nitroblue tetrazolium and 5' oromo 4 chloro 3 indoyl phosphate and stopped by incubation in 2 XSSC.

#### Detection of Chromosome Aberrations Using CISS Hybridization

Using the above-described steps, it is possible to specifically stain or label any selected individual chromosome (or chromosomes) referred to as a target chromosome, or a subregion(s) thereof. As explained in the examples, the present method has

been shown to be useful in a variety of cells, both in mitotic (e.g., metaphase, prophase) and interphase cells. As described in detail in Example 2, the CISS hybridization method of the present method is useful for rapidly screening mitotic and interphase aneuploid tumor cells for complex numerical and structural aberrations of individual chromosomes (e.g., changes in number of chromosomes, deletions and rearrangements or translocations).

In this context, biotinylated library DNA inserts were used in the CISS hybridization method to produce hybrid molecules which were detected using known techniques. Two glioma lines were used as general models of aneuploid cells, particularly tumor cells. One was an oligodendroglioma line and the other was a glioblastoma line. These were analyzed, using the biotinylated DNA probes specific for chromosome 1, 4, 7, 18 and 22. Specific labeling of the chromosomes, from pter to qter, made it possible to visualize numerical changes, deletions and rearrangements in these chromosomes in metaphase spreads and in early prophase and interphase nuclei. Complete chromosomes, deleted chromosomes and segments of translocated chromosomes were rapidly delineated in the very complex karyotypes of such cells. Additional subregional probes were also used to further define aberrant chromosomes. Digital image analysis was used to quantitate the total complement of specific chromosomal DNAs in individual metaphase and interphase cells of each line. Under-representation



of chromosome 21 and over-representation of chromosome 7 (specifically 7p) were observed. This is in agreement with previous observations by others using conventional cytogenetic banding techniques. Bigner, S.H. et al., Cancer Genet. Cytogenet., 22:121-135 (1987); Shapiro, J.R., Semin. Oncol., 13:4-15 (1986).

The two glioma cell lines used display several cytogenetic features common to many glioma cells. Thus, it is reasonable to expect that the CISS hybridization method can be used in a similar manner to specifically decorate other chromosomes and to detect those chromosomes in glial tumors. The two cell types analyzed are highly aneuploid (i.e., they have 100 chromosomes, rather than the normal 46). Therefore, it is reasonable to expect that the CISS hybridization method can be used in assessing any type of aneuploid (tumor) cell.

Thus, the CISS hybridization method can be used in assessing chromosomal aberrations associated with cancer, both in diagnosis of the disease and in monitoring its status (e.g., progression, regression or change with treatment) in patients. In this application, assessment of a single chromosome or of multiple chromosomes, and subregions thereof, can be carried out. Double hybridizations using two DNA probes, each bearing a different label can also be carried out. That is, biotinylated chromosome 7 library DNA inserts and a probe specific for alphoid repeats on chromosome 7 (pa7tl) which was modified with aminoacetylfluorene (AAF) were used to assess

chromosome 7 content/characteristics in both metaphase spreads and interphase nuclei of the two types of tumor cells (TC 593, TC 620). After hybridization, biotinylated chromosome 7 inserts were detected using avidin-FITC and chromosome 7-specific alphoid AAF labeled sequences were detected with tetramethylrhodamine isothiocyanate (TRITC) conjugated second antibodies. Double CISS hybridization was used to detect translocation between chromosome 8 and 14, Burkitt lymphoma cells, a high malignancy form of B lymphocyte tumors such were seen in both metaphase spreads and interphase cells.

This made it possible to detect similarities and differences in chromosome number 7 present in the two tumor cell types: only the four complete number 7 chromosomes found in TC 593 contained a detectable 7 centromeric signal; a smaller and metacentric number 7 chromosome lacked the 7 alphoid sequences and a small block of heterochromatin at 7q11 (indicating that it lacked a characteristic centromeric region). In contrast, all four chromosome number 7 of TC 620 were labeled with the 7 alphoid probe. Double CISS hybridization also made it possible to distinguish among number 7 chromosomes present in one cell type (TC 593) and to demonstrate similarity (at least as to the characteristics assessed) among number 7 chromosomes present in the other cell type (TC 620).

Double CISS hybridization was used to detect translocations between chromosome 8 and chromosome 14 in Burkitt's lymphoma cells; Burkitt's lymphoma

is a highly malignant form of B lymphocyte tumors. Translocations were detected in both metaphase spreads and interphase cells.

It is possible, through the use of appropriately-selected probes and/or labels to increase the number of different chromosomes, as well as the number of subregions on some or all of those chromosomes, which can be analyzed simultaneously using multiple CISS hybridization. For example, it is possible to use more than one probe, each specific for a subregion of a target chromosome, to analyze several subregions on that single chromosome at one time. It is also possible to label each probe set (set of DNA or RNA fragments) with a distinct fluorochrome or different reporter molecule, which can be distinguished from one another, after probe-target chromosome hybridization has occurred, by known techniques (e.g., by using specific fluorescent or enzyme reagents).

Furthermore, a "combinatorial" variant of CISS hybridization can be used to enhance the number of chromosomes which can be assessed simultaneously. That is, it is possible to use a hybridization probe mixture made from a single set of probe sequences composed of two halves, each separately labeled with a different fluorochrome (e.g., fluorescein and rhodamine), which, upon hybridization, produce a third fluorescence "color" or signal optically distinguishable from each of the original individual fluorochromes. Pairing of two different fluorochromes in this manner makes it possible to

identify three different chromosomes. For example, a probe set labeled only with fluorescein will yield one color upon hybridization; the same probe set labeled only with rhodamine will yield a second (different) color upon hybridization. When half of the probe set is labeled with one of the two, both sequence subsets can hybridize to target with equal probability and be perceived as a third (different) color (in a way not dissimilar to mixing paint). It is important here that two fluorochromes are not introduced into the same molecule, in order to minimize the possibility of E transfer (a well-known process where light emitted by one fluorochrome whose spectrum overlaps that of the other fluorochrome is absorbed by the second fluorochrome. The transferred electrons are emitted by second fluorochrome, which leads to quenching of the first fluorochrome. Pairwise combinations of three different fluorochromes selected for their spectral characteristics can be used singly and in pairwise combinations to produce in a similar manner. This can result in the production of six different fluorescent colors or signals (e.g., three pairs plus three single fluorochromes). Similar combinations of four different fluorochromes results in production of 10 different fluorescent colors or signals, of five different fluorochromes results in production of 15 different colors or signals, etc. This principle of combinatorial fluorescence (combining two or more fluorochromes to label the same probe set) is applicable to metaphase and

interphase chromosome analysis because each chromosome is a physically separate entity and is, thus, a distinct target. Composite probe labeling in which mixtures of three different fluorochromes are used provides even greater diversity of colors or signals useful in simultaneous multiparameter analysis.

Another approach to enhance the number of chromosomes which can be analyzed simultaneously involves a "time-resolved" method of fluorescence detection. In this instance, the DNA or RNA probes are labeled with chelating "cages" which bind specific lanthanides (e.g., Europium, terbium). Such metal chelates can be made to fluoresce. They exhibit excited state lifetimes that are much longer (micro to millisecond) than those of most normal fluorochromes (whose half lives are in the nanosecond range). Both the wavelength and the fluorescence lifetime is influenced by the nature of the lanthanide metal ion employed. If a pulsed-gating system, which excites the sample with light for a few nanoseconds and then shuts off is used, it is possible to let short-lived fluorochromes decay to their ground state, open the detector system at a defined time after excitation, (i.e., 1-100 microseconds) and detect only long-lived fluorochrome. This method can be used to discriminate 2 fluorescent dyes which have identical spectra but different lifetimes, thus adding a time factor to fluorochrome discrimination.

Another approach to increase the number of different chromosomes that can be analyzed simultaneously is based on a detection system which distinguishes chromosomes in terms of the flexibility or rigidity of an attached fluorochrome. Here, two single stranded probe sets can be labeled with the same fluorochrome, in one probe set the fluorochrome is introduced into the body of DNA sequences which will form hybrid molecules with the target DNA of interest. In the second probe set, the fluorochrome is introduced into DNA sequences, that do not hybridize with the target DNA (e.g., by adding a 3'-tail of poly dA-fluorochrome with deoxynucleotide terminal transferase, ligation of fluorochrome-labeled heterologous DNA to the probe DNA or other conventional secondary labeling techniques known in the art). Fluorochromes within the body of the DNA which form probe-target chromosome hybrids will become immobilized and thus will be unable to rotate freely in solution. In contrast, fluorochromes in the single-strand DNA that is not involved in hybrid formation are not immobilized and can rotate much more freely in solution. By measuring the rate of fluorochrome rotational freedom, (i.e., by measuring how fast the fluorochromes become depolarized when illuminated with polarized light) one can discriminate the two sets of probes.

Use of CISS hybridization and regionally defined probe sets for rapid assessment of chromosome aberrations associated with genetic disorders and chromosomal damage

It has been demonstrated that the CISS hybridization method is useful for the rapid assessment of chromosome aberrations (such as numerical and structural aberrations of chromosome 21) associated with genetic disorders (e.g., in the case of chromosome 21, Down syndrome). DNA probe sets which specifically label the terminal band 21q22.3 or decorate the entire chromosome 21 aberrations in metaphase and interphase cells are described in Example 3, the cloned DNA fragments from the human chromosome 21 are useful to specifically label the cognate chromosomal region in metaphase spreads and interphase nuclei in a variety of cell types. That is, CISS hybridization using a chromosome 21 probe set was shown to be effective in labeling/identifying chromosome 21 DNA in lymphocytes, embryonic chorionic villi cells and a glioma tumor cell line (TC 620). Unique probe sets from band 21q22.3 were also used to detect chromosome solid tissue ("normal" human brain tissue). Thus, CISS hybridization and hybridization with pools of unique sequence probes clearly have potential as a diagnostic for Down syndrome and for other genetic diseases or other conditions associated with chromosomal aberrations.

Results demonstrate that a trisomic karyotype can be diagnosed easily in interphase cells because the majority of the nuclei (55-65%) exhibit three distinct foci of hybridization. In contrast, less

than 0.2% of nuclei in lymphocytes with a disomic karyotype show three nuclear signals; interestingly, the percentage of such nuclei in normal CV cells was higher but still considerably less than 5%. In general, as few as 20-30 cells were sufficient to unambiguously distinguish between disomic and trisomic cell populations. However, in view of the uncertainty of the level of chromosome 21 mosaicism in clinical samples, the number of cells required to make an unambiguous diagnosis will likely be higher. Additional clinical correlations will be required to establish the absolute number. Nevertheless, this analytical approach could allow the diagnosis of Down syndrome without the need to culture cells or to obtain metaphase spreads. It would also decrease the time required to make the diagnosis, from the current 10-14 days to 1 day or less.

Although selected plasmid clones containing only unique human DNA sequences were used here, cosmid clones containing repetitive sequences can also be used to specifically label their cognate genomic region in metaphase and interphase cells by applying hybridization protocols like CISS hybridization that suppress the signal contribution of repetitive sequence elements. Therefore, single or nested sets of cosmids could be used as diagnostic tools for other genetic diseases in a fashion similar to that reported here. Trisomy of chromosomes 13, 18 and 21 and numerical changes in chromosomes X and Y together account for the vast majority of numerical chromosome abnormalities



identified during prenatal karyotyping. With the continued development of multiple nonisotopic probe labeling and detection systems it should be possible to visualize three or more chromosomes simultaneously following in situ hybridization. The variations, described in the previous section, of the CISS hybridization method which increase the number of chromosomes, and/or the number of chromosome regions which can be assessed simultaneously can also be used for detecting chromosomal aberrations associated with genetic disorders and chromosomal damage. Thus, the development of a rapid and automated screening test to detect the major trisomic disorders directly in interphase cells from amniotic fluid or chorionic villi cells is a viable future objective. The analysis of specific human chromosomes by in situ hybridization has already been used to complement conventional cytogenetic studies of highly aneuploid tumor lines (Example 2) and the extension to prenatal diagnostic applications seem warranted.

The analysis of karyotypes with translocations of chromosome 21 shows the usefulness of a regional probe set to rapidly identify and characterize even small translocations by unambiguous signals on metaphase chromosomes, thus circumventing an extensive analysis by high-resolution banding. In contrast, the library insert probe is more suitable for defining the relative amount of chromosome 21 DNA that has been translocated. By analyzing interphase nuclei, one can also determine if a

balanced or unbalanced number of chromosomal regions exists. However, the detection of a translocated chromosome directly in nuclei would require double-labeling techniques to identify the recipient chromosome to which the chromosome 21 material was translocated. With prior knowledge of the chromosome in question, such translocation events could be assessed by measuring the juxtaposition of the nuclear signals. Rappold, G.A. et al., Hum. Genet., 67:317-325 (1984).

A cosmid clone spanning the entire muscular dystrophy (MD) locus on chromosome X has been used to identify translocation between chromosome X and chromosome 4.

Probes containing 6 kb of sequence were localized in both metaphase spreads and interphase cells with high efficiency. This detection sensitivity with nonisotopic reagents is similar to that achieved in other recent reports. The combination of nonisotopic in situ hybridization with DAPI or BrdUrd banding or total chromosome decoration with library DNA probes thus provides a simple and general approach for gene mapping. Combinatorial fluorescent technology will also make it possible to examine several chromosomal regions simultaneously, thus permitting genetic linkage analysis by in situ hybridization. It also should facilitate the use of small DNA probes to rapidly pinpoint the breakpoints on translocation chromosomes, which could further aid in defining the genomic segments critical for Down syndrome.

Identifying and Isolating Chromosome-Specific  
Sequences Using CISS Hybridization

The CISS hybridization method of the present invention can also be used to identify chromosome-specific sequences and, subsequently, to separate them from repetitive sequences, using known techniques. Such chromosome-specific sequences, separate from the non-specific or repetitive sequences, and labeled, can be used in hybridization assays carried out, for example, in a diagnostic context, to identify, detect, and/or quantitate a chromosome or chromosome region of interest (e.g., one which is associated with a genetic disorder or causes an infectious disease). Combination of a sample to be assayed for a selected target nucleic acid sequence or sequences and appropriately-selected, labeled chromosome-specific sequences separated from repetitive sequences (e.g., sequences specific for sequences on the chromosome(s), generally referred to as target nucleic acid sequences, which are to be detected and/or quantitated in the sample under appropriate conditions results in hybridization with complementary sequences present in the sample. Hybridization will not occur, of course, if complementary sequences are not present in the sample.

Such separated chromosome-specific nucleic acid sequences can be incorporated into a kit to be used for identification, detection and/or quantitation of chromosomes or chromosome regions of interest, using

standard hybridization techniques. For example, labeled nucleic acid sequences which are chromosome 21 specific (or specific to a portion of chromosome 21), identified by CISS hybridization, and separated from repetitive sequences present on chromosome 21, can be included in a kit, along with other reagents such as buffers, competitor DNA, carrier DNA and substances needed for detection of labeled chromosome 21-derived nucleic acid sequences hybridized to chromosome 21 sequences present in a sample. Such kits clearly can be produced to include chromosome-derived nucleic acid sequences from one or more chromosome(s) of interest. Competitor DNA, carrier DNA and substances useful for detecting hybridized sequences will be as described above.

EXAMPLE 1 Cyto-Specific Staining of Individual Human Chromosomes Using Genomic DNA Libraries in CISS Hybridization

DNA libraries

The following human chromosome genomic libraries were obtained from the American Type Culture Collection: LA01NS01 (chromosome 1), LL04NS01 (chromosome 4), LA07Ns01 (chromosome 7), LL08NS02 (chromosome 8), LA13NS03 (chromosome 13), LL14NS01 (chromosome 14), LL19NS01 (chromosome 18), LL20NS01 (chromosome 20), LL21NS02 (chromosome 21), LA22NS03 (chromosome 22), LA0XNL01 (chromosome X). Amplification of these phage libraries on agar plates (using LE 392 cells as the bacterial host),

purification of the  $\lambda$  phages and extraction of phage-DNA pools were carried out according to standard protocols. Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NJ (1982).

Preparation of metaphase spreads and fibroblast cells

Phytohemagglutinin-stimulated lymphocytes from a normal adult male (46, XY) were cultured in McCoy's 5A medium (GIBCO), arrested with Colcemid, treated with a hypotonic solution of 0.075 M KCl, fixed in acetic acid-methanol and metaphase spreads made by standard procedures. Low-passage normal human foreskin fibroblasts (46, XY) were grown on microscope slides, fixed with paraformaldehyde, and permeabilized as described for study of preparations with a more intact three-dimensional structure. Manuelidis, L, Ann. NY Acad. Sci., 450:205-221 (1985).

Preparation of DNAs for in situ hybridization

Insert DNA probes. Genomic DNA fragments from the chromosomal DNA libraries were separated as a pool from the Charon 21A vector arms by digestion with the appropriate restriction enzyme [EcoRI (LA libraries) or Hind III (LL libraries)], followed by preparative electrophoresis in 0.6% agarose gel. The insert fragments were isolated from gel slices by electroelution into an Elutrap (Schleicher and

Schuell) and further purified by Elutip-d column chromatography (Schleicher and Schuell). The DNA was then extracted with phenol/chloroform (1:1) and ethanol precipitated. This pool of DNA fragments was labeled either by nick translation using Bio-11-dUTP or by random primer extension with the multiprime DNA labeling system (Amersham) substituting dTTP with 0.5 mM Bio-11-dUTP. Langer, P.R. et al., Proc. Natl. Acad. Sci. USA, 78:6633-6637 (1981) and Brigati, D.J. et al., Virology, 126:32-50 (1983). Alternatively, the DNA of the chromosome-specific libraries was biotin-labeled directly (without separation of the vector arms) by nick translation.

Probe size. To facilitate probe penetration and to optimize reannealing hybridization, it is desirable to have labeled DNA fragments smaller than 500 nucleotides; the majority of the probes are generally 150 to 250 nucleotides in length. DNase concentrations were empirically established in nick-translation reactions to yield fragments in the desired size range and this was verified by agarose gel electrophoresis. Random primer extensions were also carried out under conditions which yielded a comparable DNA size distribution.

Competitor DNA. Human genomic DNA (from placenta or white blood cells), prepared as described, as well salmon testis genomic DNA (Sigma) were digested with DNase to obtain fragments with the same size distribution as the probe DNA, then extracted with phenol/chloroform and ethanol

precipitated. Davis, L.G. et al., "Basic methods in molecular biology", Elsevier, New York Amsterdam (1986). These competitor DNAs were used in varying ratios with probe sequences, as described with reference to Figure 2.

Preannealing and hybridization. Under standard conditions, from 5  $\mu\text{g/ml}$  to 30 $\mu\text{g/ml}$  of biotin-labeled DNA, representing library insert fragments, and varying amounts of competitor DNAs were combined, ethanol-precipitated and resuspended in formamide. The probe concentration was adjusted to reflect the relative DNA content of each chromosome target. For example, chromosome 1 contains approximately 5.3 times as much DNA as chromosome 21; thus the probe concentrations used were 30  $\mu\text{g/ml}$  and 5  $\mu\text{g/ml}$ , respectively. Mendelsohn, M.L. et al., Science, 179:1126-1129 (1973). When total library DNA was used as the probe mixture instead of purified DNA inserts, 10 times as much labeled DNA was added to compensate for the large amount of vector sequences. In the case of the X-chromosome library, LA0XNL01, only twice as much labeled library DNA was used, since the human DNA inserts constitute almost half of the total DNA. For comparative purposes, the concentration of human competitor DNA in the hybridization mixture was varied from 1 to 1.0 mg/ml and salmon testis DNA was added as required to result in a final DNA concentration of 1.0 mg/ml in 50% formamide, 1 x SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and 10% dextran

sulfate. These solutions were heated at 75°C for 5 min. to denature the DNA and then incubated at 37°C for various times to promote partial reannealing. The preannealing step was done in an Eppendorf tube just prior to applying the hybridization mixture to the specimen. Nuclei and chromosome spreads on glass slides were incubated in 70% formamide, 2 X SSC] at 70°C for 2 min. to denature chromosomal DNA and then dehydrated in a series of ice-cold ethanal (70%, 90% and 100%, each for 3 min.). After application of the preannealed probe mixture (2.5  $\mu\text{l}/\text{cm}^2$ ) to slides prewarmed to 42°C, a coverslip was added and sealed with rubber cement. The samples were then immediately incubated at 37°C in a moist chamber for 10-20 h.

In those cases where paraformaldehyde fixation was used to more optimally preserve the 3-D structure of the specimen, the slides were equilibrated in 50% formamide, 1 X SSC (2 X 5 min.), excess fluid was removed without permitting the sample to dry, the probe mixture was added (5  $\mu\text{m}/\text{cm}^2$ ), and a coverslip mounted and sealed with rubber cement. Manuelidis, L., Ann. NY Acad. Sci., 450:205-221 (1985). Denaturation of both probe and cellular DNA was done at 75°C for 5 min. before hybridization was allowed to proceed overnight at 37°C.

#### Detection

After hybridization, the slides were washed in 50% formamide, 2 X SSC (3 X 5 min., 42°C) followed by washes in 0.1 X SSC (3 X 5 min., 60°C).



Thereafter the slides were incubated with 3% bovine serum albumin (BSA), 4 x SSC for approximately 30 minutes at 37°C. Detection of the biotinylated probe was achieved using either fluorescein-labeled avidin or avidin-alkaline phosphatase complexes. All detection reagents were made up in 4 X SSC, 0.1% Tween 20, 1% BSA and all washes were carried out in 4 X SSC, 0.1% Tween 20 (3 X 3 min., 42°C). For fluorochrome detection, slides were incubated with 5 µg/ml fluorescein isothiocyanate (FITC)-conjugated avidin DCS (Vector Laboratories) at 37°C for 30 min., followed by washes. In rare cases, the FITC signal was amplified by incubation with 5 µg/ml biotin-conjugated goat anti-avidin D antibodies (Vector Laboratories) at 37°C for 30 min., followed by washing, a second incubation with 5 µg/ml FITC-conjugated avidin (37°C, 30 min.) and a final wash. Pinkel, D. et al., Proc. Natl. Acad. Sci. USA, 83:2934-2938 (1986). For detection by enzyme activity, samples were incubated with 2.5 µg/ml streptavidin, washed, incubated with 2 µg/ml biotin-conjugated alkaline phosphatase (Vector Laboratories), washed again and pre-equilibrated in AP-buffer 9.5 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>) for 2 x 5 min. at room temperature. The enzyme reaction was carried out in AP buffer 9.5 containing 330 µg/ml of nitroblue tetrazolium (NBT) and 165 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at 37°C for 0.5-1 hour and stopped by incubation in 2 X SSC. All preparations were counter-stained with 200 ng/ml 4,6-diamidino-2-phenylindole-

dihydrochloride (DAPI), 2 X SSC for 5 min. at room temperature and mounted in 20 mM Tris-HCl, pH 8.0, 90% glycerol containing 2.3% of the DAPCO antifade, 1,4 diazabicyclo-2(2,2,2)octane. Johnson G.D. et al., J. Immunol. Methods, 55:231-242 (1982).

### Densitometry

A graphics workstation (VAX station II/GPX, Digital Equipment Corporation) with a frame grabber (ITEX FG-101, Imaging Technology) and a Dage-MTI-65 video camera with a Zeiss S-Planar 60 mm lens were used as described in Manuelidis, L. and J. Borden, Chromosoma, 96:397-410 (1988). Images were digitized directly from the negatives and stored on disk. Background was removed and polygonal regions around each chromosome were defined. Threshold density levels were used to outline chromosome regions within the defined polygonal areas. Means density levels within these outlined chromosome regions,  $R$ , were determined by the total signal  $\int I(x,y) dR / \text{area } R$ , where  $\int I(x,y)$  is the pixel intensity (0-225) at each point within the region  $R$ . The threshold background intensity was subtracted from the mean regional density, both for labeled chromosome 7 and for background chromosomes. The signal to noise ratio was calculated as mean chromosome 7 signal/mean background chromosome signal.

The following is a description of the results of the work described above, which clearly demonstrate specific labeling of the individual chromosome indicated. The first sections describe

use of chromosome library inserts labeled with biotin and the second describes use of DNA insert fragments.

Figure 2 shows suppression of signals from cross-reacting sequences within a chromosome 7-derived DNA library by different concentrations, as described below.

Figure 2A shows chromosome 7 library inserts labeled with biotin and hybridized to metaphase spreads from normal human lymphocytes without human competitor DNA. Prominent labeling of the two no. 7 chromosomes is observed; additionally, a distinct band-like patterns of hybridization is seen on most of the other chromosomes, and two E-group chromosomes are especially brightly stained. This general chromosomal banding pattern resembles R-banding, and suggests that a significant portion of the background cross-hybridization signal originates from Alu repetitive sequences. Previous studies have shown that Alu sequences delineate an R-banding pattern, while Giemsa positive-banding profiles are highlighted by KpnI interspersed repeats. Manuelidis, L. and C.D. Ward, Chromosoma, 91:28-38 (1984).

Establishment of experimental procedure to eliminate the hybridization signal from repetitive elements

A series of pilot studies were therefore undertaken to establish experimental parameters to eliminate the hybridization signal from such repetitive elements. The kinetics of nucleic acid

reassociation in solution are dependent on the total concentration of nucleic acid ( $C_0$ , in moles of nucleotides per liter) and the time of renaturation ( $t$ , in seconds). When reassociation conditions are standardized for temperature (taking into account the formamide concentration), cation concentration and buffer system, the reassociation kinetics are comparable with respect to  $Cot$  values. Under defined conditions, the fast reassociating fraction of mammalian genomes containing the highly repetitive DNA is completely reannealed at  $Cot$  values between  $1 \times 10^{-1}$  and  $5 \times 10^{-1}$ ; the intermediate fraction containing the middle repetitive DNA is completely renatured at a  $Cot$  value of  $1 \times 10^2$ . Britten, R.J. and D.E. Kohne, Science, 161:529-540 (1968). Thus at a human DNA concentration of 1.0 mg/ml (corresponding to  $3 \times 10^{-3}$  moles of nucleotide per liter), the fast fraction would be renatured in approximately 10s, whereas the middle repetitive DNA would need more than 9 h to reach complete reannealing. Since the fast fraction of reassociating DNA containing most or all of the ubiquitous repetitive DNA causing cross-hybridization signals, a total DNA concentration of 1.0 mg/ml was used and partial reannealing of the probe mixture was allowed prior to application to specimens. The optimal renaturation time was determined empirically (see below). This was important because the in situ hybridization conditions deviate from the standard conditions under which reassociation kinetics are determined (e.g., hybridization in 50% formamide at

37°C corresponds to 0% formamide at about 70°C; dextran sulfate also increases the reassociation time significantly). Furthermore, it was unclear to what degree the middle repetitive DNA contributed to the non-specific signal and therefore should also be prevented from hybridization by a preannealing procedure.

The stringency for the reannealing and in situ hybridization experiments was determined in 50% formamide at 37°C (adapted from standard in situ hybridization protocols) and 1 X SSC [this cation concentration of 0.165 M comes close to the concentration used in kinetics the study of Britten and Kohne. Britten, R.J. and D.E. Kohne, Science, 161:529-540 (1968)]. Competitor human DNA was added in the reassociation procedure to obtain the desired final high DNA concentration and to maintain a high level of repetition of the DNA sequences that should preanneal. While total human genomic DNA represents all the highly repetitive DNA to be removed by preannealing, it also contains sequences of the target chromosome. Thus, the addition of excessive amounts of human DNA would be expected to diminish the chromosome-specific signal. Therefore, the optimal concentration of total human DNA to use as the competitor was first determined. To keep the total DNA concentration constant at 1.0 mg/ml, genomic salmon DNA was added as needed. Salmon DNA shares certain repetitive DNA elements, such as poly dCda in common with human DNA, but lacks others, most notably the Alu- and KpnI repeats. Hamada, H. et

al., Proc. Natl. Acad. Sci. USA, 79:6465-6469 (1982). This results in a lower frequency of the latter sequences with increasing amounts of salmon DNA in the reassociation reaction.

Figure 2 shows typical experimental results obtained when 20  $\mu\text{g/ml}$  of the chromosome 7 probe set was denatured together with 50  $\mu\text{g/ml}$  (B), 100  $\mu\text{g/ml}$  (C), 200  $\mu\text{g/ml}$  (D) or 1000  $\mu\text{g/ml}$  (E) of DNase-digested human genomic DNA which was preannealed for 20 min. Hybridization and detection using avidin-FITC were carried out as described above. From each preparation ten black and white pictures were taken under standardized photographic conditions for densitometric studies (see below). In the absence of human genomic competitor (A) the signal showed little chromosomal specificity. However, with 50 and 100  $\mu\text{g/ml}$  of human competitor DNA, as increase of label specificity is readily apparent (Figure 2B,C). Specific staining of chromosome 7 was achieved with a peak of signal intensity using 100 and 200  $\mu\text{g/ml}$  of human competitor DNA (Figure 2C, D). Higher concentrations of human DNA caused an apparent decrease of signal intensity, especially at 1000  $\mu\text{g/ml}$  human DNA (Figures 2E). However, the signal obtained under these latter conditions is still reasonably bright to the observer, but requires a different exposure for optimal illustration (not shown).

A computer-assisted method of quantitative densitometry (see above) was used to establish the overall level of labeling specificity. The ratio of

[illegible]

Table 1 Densitometric analysis of the suppression of cross hybridization signals by concentrations of human competitor DNA

DNA conc. $\mu\text{g/ml}$ )	Signal- to-noise ratio				Confidence interval <sup>c</sup>
Human competitor DNA conc.	<u>Signal</u>		<u>Noise</u>		
	Pixel <sup>a</sup>	$\eta$	Pixel <sup>b</sup>	$\eta$	(99%)
0	71.48	8	54.66	26	1.31 $\pm$ 0.04
50	74.50	8	37.43	28	1.99 $\pm$ 0.07
100	162.64	8	20.06	23	8.11 $\pm$ 0.35
200	147.35	8	20.53	26	7.18 $\pm$ 0.37
500	89.78	8	18.63	21	4.82 $\pm$ 0.28
1000	94.37	8	30.51	17	3.09 $\pm$ 0.12

<sup>a</sup> Mean value of pixel intensity of target chromosome

<sup>b</sup> Mean value of pixel intensity of non-target chromosomes (from the same metaphase spreads)

<sup>c</sup> The confidence interval was calculated using Fieller's theorem (Finney, D.J., Statistical methods in biological assay, 2nd edn., Hafner Press, N.Y., 1971)

$\eta$  Number of chromosomes from which the mean was determined

Optimal reannealing conditions for suppression of nonspecific signal (using 20  $\mu\text{g/ml}$  of chromosome 7 probe and 100-200  $\mu\text{g/ml}$  human genomic (DNA), gave a



signal-to-noise ratio of ca. 8:1. Additional attempts to improve the signal to noise ratio by increasing hybridization stringencies (e.g., 60% formamide or 0.2 x SSC) gave no apparent improvement and led to an overall decrease in signal intensity.

Since about 100-200  $\mu\text{g/ml}$  of human competitor DNA was shown to give the optimal specificity, 200  $\mu\text{g/ml}$  was used for another analysis of signal specificity with respect to the renaturation time (see above). After 0, 2, 5, 10, 20, 40 and 50 min. of preannealing, aliquots were taken and used for in situ hybridization experiments as before. As indicated in Figure 3, specific labeling was obtained for all preannealing times. A small improvement of the signal is seen with increasing renaturation times from 0 to 20 min. Longer renaturation times up to 60 min. (not shown) gave no significant improvement in signal strength or chromosome specificity. The subjective impression of a signal improvement with 20 min. of preannealing (Figure 3D) could not be confirmed by a densitometric analysis, carried out as described above, since no significant differences in the signal-to-noise ratio of the different preannealing times were observed (data not shown). Therefore, the standard renaturation time in all subsequent experiments was 10-20 min. Since a signal is clearly visible at renaturation time 0, the few seconds necessary for transferring the probe mixture to the microscope slide appear to be sufficient to effectively preanneal many of the sequences that cause nonspecific labeling by

cross-hybridization. Furthermore, the large excess of single-stranded competitor DNA may efficiently compete with the biotinylated probe sequences for ubiquitous chromosomal target sites during the hybridization reactions. These results demonstrate that the majority of highly repetitive DNA sequences can be sufficiently suppressed to achieve chromosome-specific labeling by in situ hybridization.

In certain cases the signal distribution over the entire chromosome shows some variability from experiment to experiment. When the overall signal is decreased, some chromosomal subregions show a brighter staining; these signal hotspots generally constitute chromosomal sites that contain known chromosome-specific repetitive sequences. In the experiments shown in Figures 2 and 3, predominant staining of the centrometric region of chromosome 7 is seen, which corresponds to the chromosome-specific signal of an alphoid repetitive DNA. Waye, J.S. et al., Mol. Cell Biol., 7:349-356 (1987) and see Example 2. Apparently, the abundance of these repeated sequences is sufficiently low to prevent their suppression under the conditions used here. The unequal signal distribution can be overcome by amplifying the overall signal using an antibody sandwich technique as described above. Furthermore, a predominant staining of the region 1q12 that corresponds to the chromosomal site of satellite III DNA was frequently observed in labeling chromosome 1. Cooke, H.J. and J. Hindley, Nucleic Acids Res.,

6:3177-3197 (1979) and Gosden, J.R. et al., Cyto-genet. Cell Genet., 29:32-39 (1981) and see Example 2. An example of the balanced signal distribution seen after such an amplification step is shown in Figure 4A.

Several commercially available DNA libraries, each representing a single human chromosome, were tested for their ability to specifically label the chromosome they represented, under the standardized reannealing conditions described above and with the probe concentrations adjusted for chromosome size, as described above. Some examples, for chromosomes 1, 4, 7, 13, 18 and 20, as shown in Figure 4, clearly demonstrate that specific labeling can be achieved with most chromosome libraries. Table 2 lists the libraries tested with their relative scores of labeling specificity. All scores are positive because the chromosome of interest was always decorated. The highest score (4+) is used when no significant cross-hybridization to other chromosomes was observed and the scores decrease (3+ to 1+) with an increasing amount of cross-hybridizing sequences.

Table 2    Relative quality of specific chromosome  
             labeling in situ using preannealed bio-  
             tinylated library DNA inserts

Chromosome	Library used (ATCC designa- tion)	Relative specificity of in situ hybridi- zation signal <sup>1</sup>
1	LA01NS01	3+
4	LL04NS01	4+
7	LA07NS01	4+
8	LL08NS02	4+
13	LA13NS03	1+
14	LL14NS01	2+
18	LL18NS01	4+
20	LL20NS01	4+
21	LL21NS02	3+
22	LA22NS03	3+ <sup>b</sup>
X	LA0XNL01	4+

<sup>a</sup> See the text for score definition

<sup>b</sup> Under standard preannealing conditions the chromosome 22 library gave a score of +1; a value of +3 was achieved only with a human competitor DNA concentration  $\geq 700 \mu\text{g/ml}$  (total DNA concentration 1.0 mg/ml).

All attempts to reduce the additional signals on other chromosomes by varying the experimental conditions failed except in experiments with chromosome 22; in this case higher concentrations of human competitor DNA (700  $\mu\text{g/ml}$ ) resulted in a significant

improvement of signal specificity. The library exhibiting the lowest chromosome specificity was the chromosome 13 library (Figure 4E). Multiple minor binding sites on other chromosomes, as well as an exceptionally bright staining of Yq12 were observed; the signal on the Y chromosome was visible using either female or male human DNA as the competitor. None of the experimental parameters tested improved on the overall specificity of this library.

Remarkably, a weak signal or even absence of signal can be observed at the centromeric region of some chromosomes (see chromosomes 4 and 18, Figure 4C,D). In contrast to chromosomes 1 and 7, which contain chromosome-specific repetitive elements, the centromere regions of chromosomes 4 and 18 apparently contain repetitive sequences, most likely alphoid satellite DNAs, which are very abundant and thus are suppressed by the reannealing technique. However, these chromosomal regions are very small and the effect can only be observed when the corresponding chromosomes are fairly elongated.

Biotinylated total library DNA (containing the phage vector sequences) was also used as probes, in concentrations adjusted to the amount of human DNA inserts. (see above). One example is shown in Figure 4F with the chromosome 20 library. Although good staining of the chromosome of interest generally was achieved, significant nonspecific background on the entire slide was a common problem. Similar results were obtained with plasmid libraries containing human DNA subcloned from the lambda phage

libraries. In contrast, there was no background problem with the total chromosome library LA0XNL01, which contains a significantly smaller proportion of vector sequences in the probe mixture since the size of the human DNA inserts is much larger.

The suppression of repetitive sequences by this reannealing technique also permits the use of flow-sorted chromosome libraries to detect chromosomal domains within interphase nuclei. Typical examples of results obtained after hybridization of chromosome 1, chromosome 7 and chromosome 18 probe sets to normal human lymphocytes after acetic acid-methanol fixation are shown in Figure 5. Discrete focal domains of hybridization signal are seen with all libraries that had scores of 2+ or more (see Table 2).

Most nuclei ( $n \geq 100$  per estimate) exhibited two domains (60%-70%); however, a significant number showed only a single domain (20%-30%) or no hybridization signal at all (5%-10%). Accordingly, ca. 95% of male nuclei exhibited one and ca. 5% showed no hybridization signal when the X chromosome library DNA was used as probe. Notably, no nuclei with three domains were found with any of the chromosomal probe sets tested. In contrast, all metaphase spreads showed the decoration of both chromosome homologs without exception. This interphase variability may reflect, in part, the close juxtaposition of two individual domains in some cells, or the inability to resolve domains that actually occupy different areas within the nuclear

volume but are unresolved when examined by two-dimensional imaging methods (see Fig. 5D; for discussion see also Cremer et al., Exp. Cell Res., 176:199-220 (1988). The small number of nuclei exhibiting no hybridization signal may be a reflection of suboptimal hybridization conditions. It is of interest to note that the size of the intranuclear domains correlates reasonably well with the relative size of the cognate metaphase chromosome. These observations provide a definitive proof that the DNA of individual chromosomes exhibits a clear territorial organization in the interphase nucleus of a normal human cell.

Acetic acid-methanol fixed nuclear spreads, such as those shown in Fig. 5, clearly retain the territorial organization for each of the chromosomes examined; however, the nuclear structure is not optimally preserved. Additional studies with specimens that possess better preservation of 3-D structure using paraformaldehyde fixed human diploid fibroblasts and a laser-scanning confocal fluorescence microscope assembly for 3-D image reconstruction have been done. The cells were fixed and permeabilized as described by Manuelidis and hybridized with chromosome library probes as outlined above. Manuelidis, L., Ann. NY Acad. Sci., 450:205-221 (1985). The probe-competitor DNA mixture was applied directly to the slide and denatured at the same time as the cellular DNA. Results showed the arrangement of the chromosome 7 domains in the nucleus and the frequently observed

helical structure of labeled chromatic within chromosome domains. The degree to which this helicity reflects true domain substructure or is an artifact reflecting preparation and fixation procedures is currently being investigated. Nevertheless, this preliminary observations establishes the feasibility of using chromosome specific probes to analyze the topography of chromosomal domains in the interphase cells.

EXAMPLE 2 Detection of Chromosome Aberrations in Tumor Cells by CISS Hybridization Using Chromosome-Specific Library Probes  
Cells

TC 593 is a pseudotetraploid cell line (modal chromosome number, 83) established from a human glioblastoma; it grows in a flat, spreading fashion and contains many process. TC 620 is pseudotriploid with a modal chromosome number of 64 and was established from a human oligodendroglioma; it grows in an epithelial fashion. Both cell lines have been described in detail. Manuelidis, L. and E.E. Manuelidis, In: Progress in Neuropathology, Vol. 4, 235-266, Raven Press, N.Y. (1979). The present experiments made use of subclones C2B (TC 593) and C2B (TC 620) at approximately 180 passages after repeated subcloning from a single cell of the original tumor line cultured as previously described by Manuelidis and Manuelidis (see reference above). Standard hypotonic treatment and acid/methanol



fixation of the cells were employed. Cremer et al., Exp. Cell. Res., 176:199-220 (1988).

#### DNA Probes and Libraries

Phage DNA libraries from sorted human chromosomes were obtained from the American Type Culture Collection: LA01NS01 (chromosome 1), LL04NS01 (chromosome 4), LA07NS01 (chromosome 7), LL18NS01 (chromosome 18) and LA22NS03 (chromosome 22). Amplification of these libraries, isolation of human DNA inserts and biotinylation were carried out as described in Example 1. A probe specific for alphoid repeats on chromosome 7 (pa7t1) was the gift of H. Willard and specifically decorates pericentromeric heterochromatin of chromosome 7 under high stringency conditions (60% formamide). Waye et al., Mol. Cell. Biol., 7:349-356 (1987); Cremer et al. Exp. Cell. Res., 176:199-220 (1988). Some DNA probes were modified with aminoacetylfluorene (AAF); and detected as described by Cremer et al. for double labeling experiments. Landegent et al., Exp. Cell Res., 153:61-72 (1984); Cremer, R. et al., Exp. Cell Res., 176:199-220 (1988).

#### In Situ Hybridization and Detection of Hybridized Probes

CISS hybridization with biotinylated library DNA inserts and detection of hybrid molecules was generally carried out using standard conditions, as described in detail in Example 1. In double CISS hybridizations using biotinylated chromosome 7

library DNA inserts and the AAF-modified 7 alphoid probe, the latter probe was heat denatured separately and only added to the hybridization mixture at the end of the reannealing step at a final concentration of 10  $\mu\text{g/ml}$  (see Example 1).

Digital Image Analysis of Specifically Decorated Metaphase and Interphase Chromosome

A VAX station II/GPX graphics workstation (Digital Equipment Corporation) with an ITEX FG 100-Q frame grabber (Imaging Technology) were used as previously described together with a Zeiss S-Planar 60 mm lens and a Dage-MTI 65 video camera. Manuelidis, L. and J. Borden, Chromosoma, 96:397-410 (1988). Images were digitized from negatives of metaphase spreads and interphase nuclei; the background was removed and polygonal regions were defined to specifically decorated metaphase chromosomes or interphase domains (see Example 1). A scan line algorithm was used to calculate histograms within the polygonal regions. Since the value of the histograms  $H(i)$  of a particular intensity (range 0-255) within the defined regions is the number of pixels at that intensity  $i$ , the area within the region falling within an intensity range  $i_0-i_1$  is the integral of the histogram from  $i_0-i_1$ . Similarly, the 2-D integral in the region defined by the intensity range  $i_0-i_1$  equals  $\sum H(i) \cdot i \cdot i_0$  was chosen for each hybridization, in order to properly outline the decorated chromatin and distinguish this area from background regions.  $i_1$  was set to the

maximum value 255 in order to capture the entire intensity range above the threshold.

Measurements of total signal intensity versus area were designed as a control for the potential presence of variable chromosome domain extension within interphase nuclei. In interphase, a more extended chromosome domain might be expected to have a greater area (or volume) yet a lower fluorescence signal intensity per unit area. If a constant amount of hybridized DNA corresponds to a constant total fluorescence, the total signal intensity is a measure of labeled DNA content. It is also possible to measure 3-D hybridized volumes within nuclei and 3-D integrated total hybridized signals.

Manuelidis, L. and J. Borden, Chromosoma, 96:397-410 (1988). The background,  $b$ , was subtracted from the discrete 2-D integral  $\iint I(x,y)dA$  within a labeled region  $R$ , to yield the total signal:

$Sig_t = \iint I(x,y)dA - b \iint dA$ , where  $dA$  is a single pixel. Similarly, the mean intensity within the region is calculated as 2-D integral/area or  $\iint I(x,y)dA / \iint dA$ .

The following is a description of the results, with reference to the appropriate figures, of the work described above. They clearly document structural and quantitative changes in the human glioma lines, including loss and gain of entire individual chromosomes and of chromosomal subregions. They also show that it has been possible to characterize both minor and predominant karyotypic features in each cell line. All chromosomes tested to date (i.e., 1, 4, 7, 18 and 22) clearly highlighted

numerical and/or structural aberrations, some of which were subtle.

Detection of numerical and structural chromosome aberrations in metaphase spreads.

Figures 6-8 and 12 show typical metaphase spreads from the malignant glioma cell lines TC 620 and 593 after CISS hybridization with biotinylated DNA inserts from each of the human chromosomes 1, 4, 7, 18 and 22. Hybridized inserts were detected with avidin fluorescein isothiocyanate conjugates (FITC) and cells were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Chromosomes designated as "complete" had an apparently normal size, centromere index and DAPI staining pattern. Despite this designation, these complete chromosomes may contain fine structural aberrations only detectable by additional investigations (see below). Apparently complete chromosomes 1, 4, 7, 18 and 22 were observed in both TC 620 and TC 593. Additionally, other homologs of these chromosomes showed significant rearrangements and abnormalities, including translocations and deletions. The predominant numerical and structural aberrations delineated in each of these cell lines are described below. A minimum of 25 good metaphase spreads were evaluated for each glioma line and for each chromosome. These data are summarized in Fig. 9.

#### Chromosome 1

In TC 620, the oligodendroglioma line, chromosome 1 inserts decorated two apparently

complete 1 chromosomes and two marker translocation chromosomes (Figs. 6A, 6B, 9). One marker was metacentric and contained an entirely decorated 1q arm, but its p arm was from another chromosome (of unknown origin). The other marker chromosome was submetacentric and showed a small segment from another chromosome attached to the 1p arm. In both marker chromosomes breakpoints were localized close to the centromere in 1p11 or 1q11. The identification of the 1p segment was established by DAPI banding (Fig. 6B), by 5'-bromo-2'-deoxyuridine (BrdU) banding, and by hybridization with a 1p36.3 probe (data not shown); the 1p36.3 probe additionally revealed deletion of this subregion in one of the apparently complete 1 chromosomes. The overall picture was of a nearly trisomic representation of chromosome 1, with a common breakpoint, and subsequent translocation.

In TC 593, the glioblastoma line, an even more complex pattern of numerical and structural chromosome 1 aberrations was observed. In a sample of 50 metaphase spreads, the majority (52%) showed six aberrant chromosomes that were decorated; 14% of the metaphases showed five aberrant chromosomes, and 34% showed higher numbers of chromosome 1 segments (up to 14). Figures 6C, D and 9 show the most typical, predominant karyotype and demonstrates the rapid definition of chromosome 1 abnormalities in this cell line. Aberrations included three acrocentric chromosomes with a consistent breakpoint in 1p1, chromosomes with a deletion of the distal

park of 1q, a submetacentric translocation chromosome with a loss of the complete 1q, and an iso(1p) marker chromosome (see Fig. 9).

#### Chromosome 4

In TC 620, chromosome 4-specific inserts decorated one apparently complete chromosome 4, and three additional chromosomes with segments containing chromosome 4 DNA (Figs. 7F, 9). These latter segments on translocation chromosomes would have been difficult to rapidly and unambiguously define with banding procedures alone. The smallest of the translocated chromosome 4 segments formed part of an approximately metacentric chromosome. The two larger segments were found on submetacentric chromosomes of different overall size. In the smaller chromosome, the short arm and part of the long arm of 4 were present with an apparent breakpoint at 4q2, i.e., 4pter-4q2. In the larger submetacentric chromosome, a region that may represent the rest of 4 (4q2-qter) appears. Thus the predominant karyotype of TC 620 showed only slightly more than two equivalents of chromosome 4 (see also the area measurements described below). The non-4 regions have not been further defined.

In TC 593, there were generally only two chromosomes decorated by chromosome 4 DNA inserts, and both of these were compatible with normal 4 chromosomes. Approximately 30% of the metaphase spreads in TC 593 showed an additional submetacentric chromosome with chromosome 4 material

(Fig. 7E). Thus, although both 4 chromosomes were apparently normal, there was a significant under-representation of this chromosome in this pseudotetraploid line (Fig. 9).

#### Chromosome 7

Three complete 7 chromosomes, and one smaller metacentric chromosome containing translocated 7 material were typically found in TC 620 metaphase spreads (Figs. 8A, 9). The translocated chromosome 7 material included the short arm of chromosome 7 (as shown by DAPI banding; cf. Fig. 8B) and the pericentromeric heterochromatin with the breakpoint in 7q1 (see also below).

In TC 593, five chromosomes entirely decorated by chromosome 7 insert probes were regularly observed (Figs. 7G, 8E). Four of these appeared to represent complete number 7 chromosomes, whereas one was smaller and metacentric. DAPI banding (Fig. 8E, insert) and size measurement (cf. Fig. 13) were consistent with an iso(7p). This conclusion was further supported by double in situ hybridization experiments with biotinylated chromosome 7 inserts (detected with avidin FITC) and chromosome 7-specific alphoid AAF labeled sequences (detected with tetramethylrhodamine isothiocyanate (TRITC) conjugated second antibodies). They showed that only the four complete 7 chromosomes, contained a detectable 7 centromeric signal (metaphase, Fig. 7G,H; interphase, Fig. 7I, J). Thus, the iso(7p) marker chromosome did not have a characteristic

centromeric region as it lacked both the 7 alphoid sequences and a small block of heterochromatin at 7q11 (see Fig. 8E, insert). In contrast, all four 7 chromosomes of TC 620 were labeled with the 7 alphoid probe (data not shown).

#### Chromosome 18

In TC 620, two apparently complete 18 chromosomes and a truncated minute chromosome were entirely decorated (Figs. 8C, D, 9). This truncated chromosome is 18q- (and possibly also 18p-). The rest of the chromosome 18 region(s) was never detected.

Three translocation chromosomes involving chromosome 18 material were typically detected, in addition to an apparently normal chromosome 18 in TC 593 metaphase spreads (Figs. 8F, G, 9). In a minor proportion of metaphases there was a small additional translocation observed. The exact chromosomal region from which this translocated 18 material derived could not be resolved by DAPI staining. The predominant karyotype for 18 is therefore close to tetrasomic in this cell line, but is under-represented in the pseudotriploid TC 620.

Both the 18q-marker chromosome in TC 620 and the three translocated 18 chromosomes in TC 593 hybridized strongly to a chromosome 18-specific alphoid repeat. Accordingly, both intact and aberrant 18 chromosomes could also be counted after in situ hybridization with this centromeric probe. Cremer, T. et al., Exp. Cell Res., 176:199-200



(1988) (see also below). DAPI banding and hybridization to 18-specific alphoid repeats indicated that these translocation chromosomes include the entire 18q region and the centromere, with breakpoints in 18p.

#### Chromosome 22

Two apparently normal 22 chromosomes were visualized in most TC 620 and TC 593 metaphase spreads (Fig. 10E). It was difficult to ascertain small translocations of this chromosome since hybridization with chromosome 22 inserts resulted in some cross-hybridization to other chromosomes. Some of this cross-hybridization is probably due to shared sequences from the nucleolus organizer regions (on five normal acrocentric human chromosomes) and to shared sequence motifs at the centromeres. McDermid, H.E. et al., Chromosoma, 94:228-234 (1986) and see Example 1. Finally, it should be noted that in contrast to conventional banding analysis, the current experimental approach clearly delineates numerical and structural chromosome aberrations in metaphase spreads of very poor quality (Fig. 10G, H) or in early prophase nuclei (Fig. 10A). These preparations are not accessible to banding analysis, as the chromosomes extensively overlies each other.

Evaluation of Chromosome Domains in Interphase Nuclei

One potential advantage of in situ methods is that individual human chromosomes may be directly visualized as discrete territories in interphase nuclei and thus can be of value in the analysis of solid tumor specimen. Manuelidis, L., Hum. Genet., 71:288-293 (1985); Schardin, M. et al., Hum. Genet., 71:281-287 (1985); Pinkel, D. et al., Proc. Natl. Acad. Sci. USA, 83:2934-2938 (1986). This feature of nuclear topography, also apparent in the malignant cells examined here (Figs. 7A-D, I, 10B-F), was evaluated for its accuracy and diagnostic usefulness. Figure 10A shows three apparently complete 7 chromosomes and one translocated 7p arm in a prophase TC 620 nucleus. Figures 3, and 10B show five chromosome 7 domains in interphase nuclei of TC 593, as previously depicted in metaphase spreads. Figure 10C shows a TC 620 interphase nucleus with two 18 domains of comparable sizes to those seen in normal diploid nuclei (see Example 1). A third, appreciably smaller, decorated 18 domain was also detected and represents the truncated 18 chromosomes seen in metaphase spreads described above. Figure 10D shows four chromosome 18 domains in an interphase nucleus of TC 593, which again is comparable to the numbers in metaphase nuclei. Figure 10E shows a TC 620 interphase nucleus with four chromosome 1 domains, while Figure 10F shows a TC 593 nucleus with at least five separate chromosome 1 domains (compare Fig. 6A, B and C,D, respectively).

While the hybridization patterns of nuclei shown in Figure 10 were highly characteristic for each cell line, counts of interphase chromosome domains have some inherent difficulties. As an example, Fig. 11A (dark columns) presents an analysis of the counts of labeled interphase domains in randomly selected nuclei of diploid human lymphocytes hybridized with 7 library inserts. Although the number and relative size of chromosome specific domains can be accurately assessed in the majority of nuclei, not all nuclei present a reliable index of the chromosomal constitution, since a considerable fraction of nuclei reveal only one decorated domain and occasional nuclei show no signals. Furthermore all domains are not always cleared separable in these 2-D preparations.

Figure 11 shows representative counts of these preparations. In agreement with TC 593 metaphase counts of chromosome 4, nuclear counts generally showed two clearly separated domains (Fig. 11E). However, the percentage of two-signal preparations was smaller in interphase than in metaphase (45.3% vs. 64%). This artifactual decrease was largely due to a corresponding increased percentage of nuclei showing only one decorated domain or no signal at all. Counts of zero or one domain were not present in metaphase spreads. Significantly, 19.3% of the interphase TC 593 nuclei displayed three clearly separated chromosome 4 domains, and these extra domains were not present in interphase nuclei of diploid human lymphocytes hybridized to this or

other libraries under the same conditions (Fig. 10A; Example 1). Finally, the ratio of two versus three domains was identical for both metaphase and interphase cells. Thus interphase nuclei can be reliably used for the detection of extra copies of a single chromosome or chromosomal segment but have limited reliability for detecting the loss of chromosome copies.

In situ hybridization of probes from subregions of interphase chromosomes may more accurately reflect general counts of chromosomal constitution than library probes (Fig. 11A), provided they are done under appropriately high stringency conditions Rappold, G. et al., Hum. Genet., 67:317-325 (1984); Cremer, T. et al., Hum. Genet., 74:346-352 (1986); Cremer, T. et al., Exp. Cell Res., 176:199-220 (1988). However, such regional segment probes do not delineate translocated elements or aberrant chromosomes that lack this segment. Therefore such probes are also not entirely accurate. For example, counts of chromosomes 1 in TC 620 and TC 593 with a probe specific for 1q12 indicated fewer 1 chromosomes than shown here with CISS hybridization (Fig. 9). Cremer, T. et al., Exp. Cell Res., 176:199-220 (1988). Counts of chromosome 7 using only a centromeric sequence further emphasize this point (see above). Double in situ hybridization with the AAF-modified 7 alphoid probe and biotinylated chromosome 7 library inserts typically showed interphase nuclei with five domains, of which only four were simultaneously labeled by the centromeric

probe (Figs. 7I, J, 11C). In TC 620, however, both probes gave identical results (Fig. 11B).

Over Representation and Under Representation of Specific Chromosomes

The relative chromosomal dosage in these glioma lines, was also assessed with particular interest in chromosome 7, which has been noted to be generally over-represented in gliomas. Bigner, S.H. *et al.*, Cancer Genet. Cytogenet. 29:165-170 (1986); Shapiro, J.R., Semin. Oncol., 13:4-15 (1986). For comparison, other individual chromosome probes were used as controls. Metaphase chromosome counts have shown that TC 620 is pseudotriploid with a modal number of 64 chromosomes, while TC 593 is pseudotetraploid with a modal number of 83. Manuelidis, L. and Manuelidis, E.E., IN: Progress in Neuropathology, Vol. 4, pp 235-266, Raven Press, N.Y. (1979). Accordingly, a chromosome and its segments together would be present in a balanced state if three complete copies were present in TC 620, and four in TC 593.

A relative over-representation is present if more than these respective copy numbers can be demonstrated. A number lower than the expected (trisomic or tetrasomic) value indicates that the chromosome is relatively under-represented in the karyotype. In cases where additional DAPI banding information was sufficient to define the selectively decorated abnormal chromosome, the chromosome pieces labeled by the chromosome-specific inserts were put

together for analysis (Fig. 9). In the second approach, computer analyses were used to independently verify these results (see below).

TC 620 analyzed by banding showed the equivalent of three 1 chromosome and thus indicated a balanced state for this chromosome. The same was true for the 1p arm in TC 593 which was present in four copies. However, the distal part of 1q was under-represented in TC 593 (see the detailed description given above). In both glioma lines, 7q appeared to be balanced, while 7p was over-represented once in TC 620 and twice in TC 593. Additionally, in both glioma lines chromosome 22 was clearly under-represented. In order to confirm this finding, double in situ hybridization with inserts of chromosomes 7 and 22 was performed. An example of this is shown in Fig. 12 and demonstrates over-representation of 7 DNA and under-representation of 22 DNA in the same cell. Metaphase counts done in both cell lines by this method of analysis are depicted for chromosome 7 in Fig. 11B, C (dark column) and for chromosome 22 in Fig. 11E. In summary, these two gliomas both show relative under-representation of chromosome 22 and over-representation of the 7p arm. The significant under-representation of chromosome 4 in TC 593, and a portion of 4 in TC 620 is also notable.

Digitized images were also used to quantitatively measure decorated areas in metaphase preparations and in interphase cells where chromosomal domains were well resolved.

Quantitative evaluation of chromosome equivalents (Table 3) indicated highly concordant numbers for interphase versus metaphase in 5 of 6 examples; only in TC 593 decorated with 18 inserts was there a discrepancy. This may be due to the small sample size.

Table 3.

Twenty-four metaphase spreads showing the predominant number of chromosomes decorated with DNA inserts from libraries of chromosomes 4, 7 and 18 were compared to twenty-eight interphase nuclei with well-separated domains using the same probes. Images were taken under identical (linear film) conditions and digitized. In each metaphase spread, areas obtained for each normal and aberrant chromosome were divided by the mean area obtained for  $n$  apparently complete chromosomes. In interphase nuclei, domains were compared assuming that the largest  $n$ -labeled domains represented complete (normal) interphase chromosomes. Thus the sum of these normalized values represents a measure of the number of specific chromosomes equivalents in a single cell. The mean values of several cells are shown for each case. The mean numbers of chromosomes equivalents obtained for interphase and metaphase cells show a strong overall correlation coefficient of  $r = + 0.95$ . Compared with area measurements, the mean numbers of chromosomes equivalents determined by 2-D intensity integrals

(See above) showed an overall correlation coefficient of  $r = + 0.99$ .

Table 3. Mean number of chromosome equivalents measured by digital image analysis in malignant glioma cell lines after CISS hybridization

Chromosome	Cell Line	Chromosome equivalents		
		Interphase	Metaphase	Expected
4	TC593	2.0	2.0	4.0
	TC620	2.5	2.4	3.0
7	TC593	4.4	4.6	4.0
	TC620	3.5	3.3	3.0
18	TC593	3.0	3.6	4.0
	TC620	2.5	2.3	3.0

Chromosome equivalents derived from digital image analysis independently confirm the relative representation of target chromosomes noted in both glioma lines by DAPI banding. The segments that comprise the total metaphase signal are further detailed graphically in Fig. 13. Computer analysis was especially useful in cases where the breakpoints involved in translocated segments could not be unambiguously defined. They were also of value in a quantitative assessment of interphase-metaphase correlations, and of normal and aberrant chromosomes with distinctly different sizes.



EXAMPLE 3 Rapid Detection of Human Chromosome 21  
Aberrations By In Situ Hybridization

DNA Probes

All plasmids contain inserts of human chromosome 21 that were mapped to 21q22.3. Moisan, J.P., Mattei, M.G., Baeteman-Volkel, M.A., Mattei, J.F., Brown, A.M.C., Garnier, J.M., Jeltsch, J.M., Masiakowsky, P., Roberts, M. & Mandel, J.L. (1985) Cytogenet. Cell Genet. 40, 701-702 (abstr.). Tanzi, R., Watkins, P., Gibbons, K., Faryniarz, A., Wallace, M., Hallewell, R., Conneally, P.M. & Gusella, J. (1985) Cytogenet. Cell Genet. 40, 760 (abstr.). Van Keuren, M.L., Watkins, P.C., Drabkin, H.A., Jabs, E.W., Gusella, J.F. & Patterson, D. (1986) Am. J. Hum. Genet. 38, 793-804. Nakai, H., Byers, M.G., Watkins, P.C., Watkins, P.A. & Shows, T.B. (1987) Cytogenet. Cell Genet. 46, 667 (abstr.). Munke, M., Foellmer, B., Watkins, P.C., Cowan, J.M., Carroll, A.J., Gusella, J.F. & Fracke, U. (1988) Am. J. Hum. Genet. 42, 542-549. All inserts were either known or verified by Southern blot analysis to be single-copy DNA: the plasmids other than pS2 are subclones derived from a  $\lambda$  phage library or a cosmid library. Van Keuren, M.L., Watkins, P.C., Drabkin, H.A., Jabs, E.W., Gusella, J.F. & Patterson, D. (1986) Am. J. Hum. Genet. 38, 793-804. Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A. & Chambon, P. (1982) Nucleic Acids. Res. 10, 7895-7903. Watkins, P.C., Tanzi, R.E. Gibbons, K.T., Tricoli, J.V., Landes, G., Eddy, R., Shows,

T.B. & Gusella, J.F. (1985) Nucleic Acids Res. 13, 6075-6088. Watkins, P.C. Watkins, P.A., Hoffman, N. & Stanislovitis, P. (1985) Cytogenet. Cell Genet. 40, 773-774 (abstr.). The plasmids are listed in Table 4 with the Human Gene Mapping Workshop symbols and the approximate insert fragment length. Kaplan, J.C. & Carrit, B. (1987) Cytogenet. Cell Genet. 46, 257-276.

Table 4. Plasmids with inserts from 21q22.3

		Insert length kb			Insert length kb
Plasmid			Plasmid		
BCEI	pS2 (23)	0.6	D21S56	pPW520-10R	4.6
D21S3	pPW231F	0.8		pPW520-11R	1.8
	pPW231G	0.7	D21S57	pPW523-10B	6.5
D21S23	pPW244D	1.0		pPW523-1H	7.0
D21S53	pPW512-6B	3.0		pPW523-5R	2.2
	pPW512-8B	3.8		pPW523-10R	3.8
	pPW512-1H	2.9		pPW523-19R	2.5
	pPW512-16P	2.7	D21S64	pPW551-8P	1.9
	pPW512-18P	1.6		pPW551-12P	4.2
	pPW512-4R	4.7	D21S71	pPW519-10P	0.8
	pPW512-12R	2.0		pPW519-11P	3.0
D21S55	pPW518-4H	1.6		pPW519-1R	6.0
	pPW518-10P	2.9		pPW519-8R	2.9
	pPW518-5R	5.2		pPW519-9R	1.7
D21S56	pPW520-5B	5.0		pPW519-14R	4.0
	pPW520-6B	1.0		pPW519-22R	1.8

Preparation of plasmid DNA was according to standard protocols. Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY). Various probe sets were obtained by pooling plasmids (equal molar amounts), resulting in DNA probe complexities of 95 kb (all plasmids listed). 75 kb (plasmids labeled with an asterisk), or 29 kb (plasmids labeled with a dagger).

The human chromosome 21 genomic library LL21NS02 was obtained from the American Type Culture Collection and amplified on agar plates as recommended. Phage DNA was prepared and digested with HindIII, and the DNA inserts were separated from the vector arms by preparative gel electrophoresis in 0.6% agarose. DNA was isolated from gel slices by electroelution; purified by Elutip-d chromatography. (Schleicher & Schuell); extracted with phenol/chloroform, 1:1 (vol/vol); and precipitated with ethanol.

#### Human Cells

Metaphase spreads and interphase nuclei were prepared from (i) lymphocyte cultures of normal (46, XY) individuals, (ii) lymphocytes of Down syndrome (47, +21) individuals, (iii) chorionic villi samples cultured for prenatal diagnosis (ii and iii were provided by T. Yan-Geng, Yale University Cytogenetics Laboratory), and (iv) cultures of

TC620, an oligodendroglioma-derived pseudotriploid cell line. Manuelidis, L. & Manuelidis, E.E. (1979) in Progress in Neuropathology, ed. Zimmerman, H.M. (Raven, Press New York), Vol. 4, pp. 235-266. Standard techniques of colcemid treatment, hypotonic treatment, and methanol/acetic acid fixation were used. Biopsy material from the cortical region of a "normal" human brain (46, XX) was fixed, sectioned, and permeabilized as described. Manuelidis, L. & Borden, J., Chromosoma, 96:397-410(1988).

#### In situ Hybridization

Various combinations of plasmid DNA, labeled with Bioll-dUTP by nick-translation, were used for hybridization at concentrations ranging from 2 to 15  $\mu\text{g/ml}$  depending on the pool size. Brigati, D.J., Myerson, D. Leary, J.J., Spalholz, B., Travis, S.Z., Fong, C.K.Y. Hsiung, G.D. & Ward, D.C. (1983) Virology 126,32-50. For example, 15  $\mu\text{g/ml}$  was used when the probe mixture contained 94 kilobases (kb) of insert DNA; the probe concentration was decreased in proportion to the sequence complexity of the probe mixture. The size of the probe DNA was adjusted to a length of 150-250 nucleotides empirically by varying the DNase concentration in the nick-translation reaction. The hybridization cocktail also contained 50% formamide, 0.30 M NaCl, 0.03 M sodium citrate (pH7), 10% (wt/vol) dextran sulfate, and on occasion 0.5 mg of sonicated salmon sperm DNA per ml. Simultaneous denaturation of probe and target DNA was carried out at 75°C for 6

min (metaphase spreads) or 94°C for 11 min (tissue slices). Hybridization reactions were incubated at 37°C overnight.

Delineation of individual chromosomes with DNA probes derived from sorted human chromosomes was done by (CISS) hybridization as described above. Briefly, biotinylated chromosome 21 library DNA inserts (5µg/ml), DNase-digested human genomic DNA (200µg/ml), and salmon sperm DNA (800µg/ml) were combined in the hybridization solution, heat-denatured, and partially prehybridized for 10-30 min at 37°C before application to a separately denatured specimen.

Posthybridization washes, detection of hybridized probe by using either alkaline phosphate-conjugated avidin or fluorescein isothiocyanate-conjugated avidin, and photographic conditions were as described in Example 1. When probe sets containing 29 kb or less of target sequence were used, the fluorescein isothiocyanate detection was generally enhanced by one cycle of signal amplification as described in Example 1.

All quantitative analyses of interphase signals were carried out by using slides from several independent experiments, with more than 100 nuclei being analyzed per slide. Comparison of signals in normal and trisomic samples was done in a blind-study fashion.

This work demonstrated that CISS hybridization, under the conditions described, resulted in rapid detection of numerical and structural aberrations of

chromosome 21 in both metaphase and interphase cells.

Use of Cloned DNA Fragments From Human Chromosome 21 to Specifically Label Chromosomes in Lymphocyte Metaphase Spreads and Interphase Nuclei

The maximal amount of unique sequence DNA in the probe set was ca94 kb; this probe set resulted in a clearly visible labeling of the terminal region of both chromatids of the chromosome 21 homologs (see Fig. 14B). These signals were seen unambiguously and without exception in all metaphase spreads, even in spreads of poor quality or from prophase cells (not shown). In normal interphase cells, the majority (65-75%) of nuclei exhibited two signals (see Fig. 14C), 25-30% showed one signal, and less than 5% showed no signal. Nuclei with three signals were found only rarely (<0.2%) and may reflect incomplete hybridization to a few tetraploid cells in the sample. Similar results were obtained with probe sets containing 29 or 75 kb of DNA. With probe sets containing fewer than 20 kb of insert DNA, there were increased numbers of cells with less than two signals. Thus, these probe sets were deemed unsuitable for diagnostic purposes. However, such probes still yielded specific signals on the majority of chromosomes 21, even with a 6-kb single-copy DNA (see Fig. 14A), especially when signal amplification was used.

Use of chromosome library DNA CISS hybridization for  
detecting chromosome 21

Chromosome 21 was specifically and entirely decorated in normal lymphocyte metaphase spreads, although some additional minor binding sites were seen at or near the centromeric region of other acrocentric chromosomes, especially chromosome 13 (normal karyotype not shown; Fig. 14F). Suppression with additional DNA including a plasmid L1.26, which detects a repetitive DNA located predominantly at the centromeric region of chromosomes 13 and 21, did not efficiently suppress the minor non-21 chromosomal signals. Devilee, P., Cremer, T., Slagboom, P., Bakker, E., Schoil, H.P., Hager, H.D. Stevenson, A.F.G., Cornelisse, C.J. & Pearson, P.L. (1986) Cytogenet. Cell Genet. 41,193-201. Quantitative evaluation of interphase nuclei signals again showed a negligible portion of nuclei with three signals; however, a significant increase in nuclei with less than two signals was observed (50-60% with two signals, 35-45% with one signal, and 5-10% without a signal). The numerical differences observed with the two different probes can be explained in part by the number of nuclei (up to one of three) that were excluded from the latter analysis because they exhibited larger and more diffuse signals, most likely from more than one chromosome that could not be resolved unambiguously as two separate chromosome domains in a two-dimensional representation. The minor cross-hybridizing sites noted above presented a

second experimental complication but did not adversely influence data interpretation.

#### Testing of Cells Containing Chromosome 21 Aberrations

The optimal (94 kb) plasmid pool as well as CISS hybridization with chromosome 21 library inserts were tested further by using cells containing chromosome 21 aberrations. Both probe sets permitted a fast and unambiguous diagnosis of trisomy 21 in all metaphase spreads from Down syndrome lymphocyte cultures (see examples in Fig. 14 D and E). Furthermore, the quantitative distribution of hybridization signals in interphase nuclei of the same preparation, analyzed as described above, was similar with either type of probe [ $<5\%$  of cells with no signal, 5-15% with one signal, 25-35% with two signals, and 55-65% with three signals (Fig. 14 F-J)]. Although the library DNA inserts gave up to 15% of four-signal nuclei (compare Fig. 14 F and G), most likely due to the minor binding sites on other chromosomes, the plasmid pool revealed only a negligible percentage of nuclei ( $<0.2\%$ ) with four signals. These results indicate that trisomy 21 can be detected in a diagnostically meaningful way with small populations of nonmitotic cells.



Localization of Chromosome 21 DNA in Embryonic  
Chorionic Villi Cells

Embryonic chorionic villi (CV) cells were also investigated with the 94 kb plasmid probe sets in a case where the father had a reciprocal t(4:21) translocation. Hybridization to metaphase spreads of the CV cells showed that the translocated chromosome (4pter→4q33::21q11.2→21qter) was indeed inherited by the fetus (see Fig. 13 L and M). The signals in the interphase cell nuclei (see Fig. 14K) of the CV cells had a distribution that paralleled that of cells with a normal karyotype (see above), indicating a balanced representation of 21q22.3 and excluding Down syndrome as a possible diagnosis. A small increase of nuclei with three and four signals (both <5%) over that of normal lymphocytes was also observed, probably reflecting a higher portion of tetraploid cells in such CV samples.

Localization of Chromosome 21 DNA in of Glioma Tumor  
Cells

The diagnostic potential of the chromosome 21 probes was further tested by using a glioma tumor cell line, TC620, known to be pseudotriploid with a highly rearranged genome. Cremer, T. et al., Exp. Cell Res., 176:199-220 (1988); Cremer, T. et al., Hum. Genet., In Press, (1988); Manuelidis, L. and E.E. Manuelidis, In: Progress in Neuropathology, 4:235-266 (ed. Zimmerman, H.M.) (1979). The metaphase spreads revealed two apparently normal

chromosomes 21 and one translocation chromosome (see Fig. 14 N and O). Interestingly, the chromosome 21 DNA on the translocation chromosome labeled by the library probe has a size equivalent to a normal 21q region, thus suggesting a Robertsonian translocation event. However, fine structural aberrations of 21q (i.e., small deletions, etc.) cannot be excluded by this analysis. The interphase signals seen with both the plasmid probe set and the library inserts were consistent with trisomy 21q22.3 and trisomy 21, respectively.

#### Localization of Chromosome 21 in DNA Sequences in Solid Tissues

The ability of the 94 kb plasmid probe set to localize chromosome 21 DNA sequences in solid tissues was also assessed. Both chromosomes 21 were clearly labeled by the probe, and located near the nucleolus; this nuclear location is consistent with the fact that chromosome 21 contains a ribosomal gene cluster that is usually localized in the nucleolus. This observation suggests that these probes may also prove useful for evaluating the frequency of chromosome 21 mosaicism in specific cell or tissue types. In addition, it should be of interest to see if the various karyotypic changes associated with the Down syndrome phenotype alter the normal nuclear topography of chromosome 21 in neuronal tissue.



Claims

1. A method of labeling individual mammalian chromosomes in mitotic cells or in interphase cells by in situ hybridization with chromosome-specific probes, to produce a chromosome-specific signal.
2. A method of labeling individual human chromosomes in mitotic cells or in interphase cells by in situ hybridization with chromosome-specific probes, to produce a chromosome-specific signal.
3. A method of producing highly specific decoration of an individual human target chromosome, comprising in situ suppression hybridization of labeled DNA probes, which are chromosome specific, to DNA in human mitotic cells or in human interphase cell.
4. A method of Claim 3, wherein the DNA probes are selected from the group consisting of total recombinant library DNA, DNA inserts purified from a chromosome-derived recombinant DNA library, and specific DNA fragments derived from chromosomes.
5. A method of Claim 4, wherein the labeled DNA probes are selected from the group consisting of: DNA probes labeled with at least one

fluorochrome; DNA probes labeled with at least one member of a specific binding pair; and DNA probes labeled with an enzyme.

6. A method of Claim 5, wherein the fluorochrome is selected from the group consisting of fluorescein, rhodamine, Texas red, Lucifer yellow, phycobiliproteins and cyanin dyes and the member of a specific binding pair is biotin.
7. A method of assessing chromosome aberrations in human cells by chromosomal in situ suppression hybridization.
8. A method of Claim 7, wherein the human cells are selected from the group consisting of metaphase cells, prophase cells and interphase cells.
9. A method of detecting chromosome aberrations in human aneuploid cells, comprising
  - a) combining
    - 1) the human aneuploid cells, treated so as to render nucleic acid sequences present available for hybridization with complementary nucleic acid sequences; and
    - 2) a hybridization mixture comprising labeled human DNA derived from a specific chromosome; competitor DNA; and nonhuman genomic DNA, under conditions appropriate

for hybridization of complementary nucleic acid sequences to occur; and

b) detecting labeled human DNA derived from the specific chromosome hybridized to nucleic acid sequences from the aneuploid cells.

10. A method of Claim 9, wherein the aneuploid cells are human tumor cells.
11. A method of Claim 10, in which the human tumor cells are selected from the group consisting of: metaphase cells, prophase cells and interphase cells.
12. A method of Claim 10, wherein the human tumor cell is a human glioma cell.
13. A method of detecting in a sample numerical alterations in a human chromosome present in the sample, comprising:
  - a) combining
    - 1) the sample, treated so as to render nucleic acid sequences present in the sample available for hybridization with complementary nucleic acid sequences; and
    - 2) a hybridization mixture comprising labeled human DNA derived from the selected chromosome; competitor DNA; and nonhuman genomic DNA, under conditions appropriate for hybridization of

complementary nucleic acid sequences to occur; and

b) detecting labeled DNA derived from the selected chromosome hybridized to nucleic acid sequences present in the sample.

14. A method of Claim 13, wherein the selected human chromosome ~~is~~ selected from the group consisting of the following chromosomes: 13, 18, 21, X and Y.
15. A method of Claim 13, wherein the selected human chromosome ~~is~~ chromosome number 21 and the labeled human DNA derived from the selected chromosome is DNA inserts purified from a chromosome-derived recombinant DNA library.
16. A method of determining over-representation or under-representation of a selected chromosome or a portion thereof in human tumor cells, comprising the steps of:
  - a) combining
    - 1) human tumor cells, treated so as to render nucleic acid sequences present in the cells available for hybridization with complementary nucleic acid sequences; and
    - 2) a hybridization mixture comprising labeled DNA fragments derived from a selected chromosome; competitor DNA; and nonhuman genomic DNA, under conditions appropriate for hybridization of

complementary nucleic acid sequences to occur; and

b) detecting labeled human chromosome-specific DNA fragments hybridized to nucleic acid sequences from the tumor cells.

17. A method of identifying chromosome-specific DNA present in a selected mammalian chromosome, comprising:

- a) combining the following substances:
- 1) the selected mammalian chromosome;
  - 2) DNA fragments derived from the selected mammalian chromosome bearing a detectable label;
  - 3) competitor DNA; and
  - 4) carrier DNA,
- under conditions appropriate for hybridization of complementary nucleic acid sequences to occur; to form a complex of the DNA fragments bearing a detectable label with the selected mammalian chromosome; and
- b) detecting complexes formed in step (a).

18. A method of Claim 17, further comprising isolation of chromosome-specific DNA in a selected mammalian chromosome by separating the selected complexes formed from the remaining substances combined in step (a).



DELINEATION OF INDIVIDUAL HUMAN CHROMOSOMES  
IN METAPHASE AND INTERPHASE CELLS BY IN SITU  
SUPPRESSION HYBRIDIZATION

Abstract of the Disclosure

A method of specifically decorating selected mammalian chromosomes and of detecting, identifying and and/or quantitating selected individual chromosomes, by means of chromosomal in situ suppression (CISS) hybridization. The method is useful in analyzing cells for the occurrence of chromosomes, chromosome fragments or chromosome aberrations.

Figure 1  
Outline of CISH Hybridization for  
Specific Staining of Human  
Chromosomes

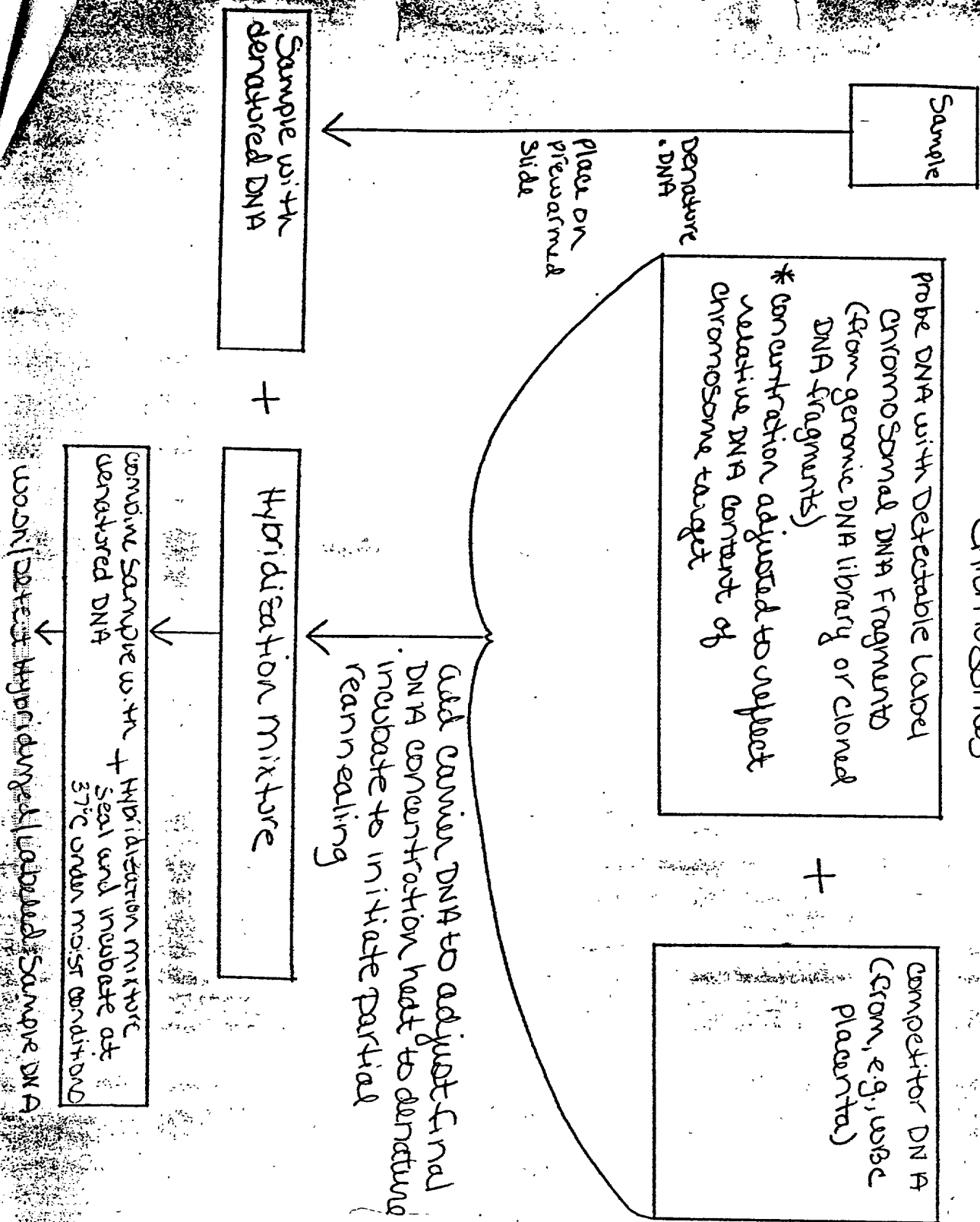
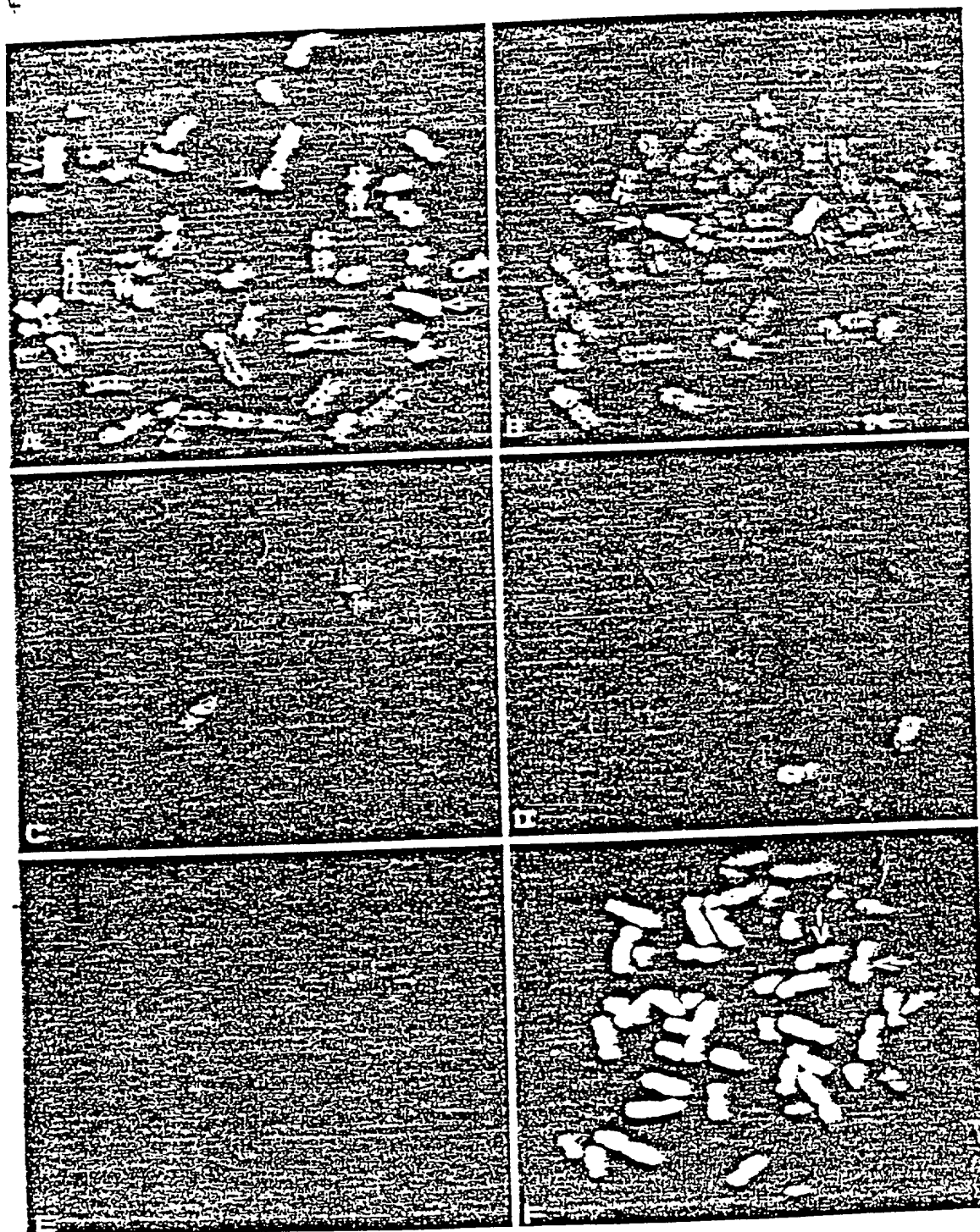


FIGURE 2



[illegible]

FIGURE 4

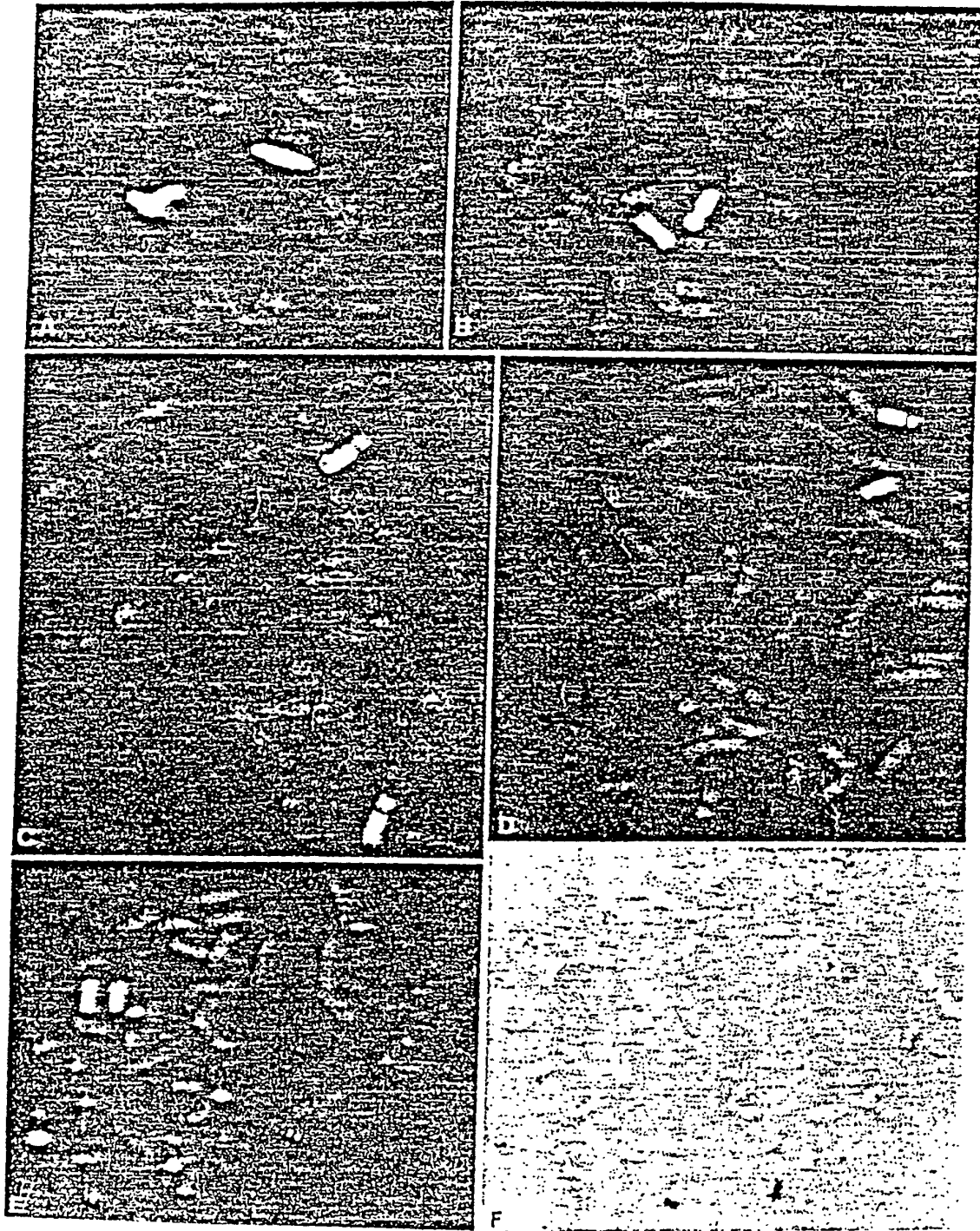


FIGURE 5

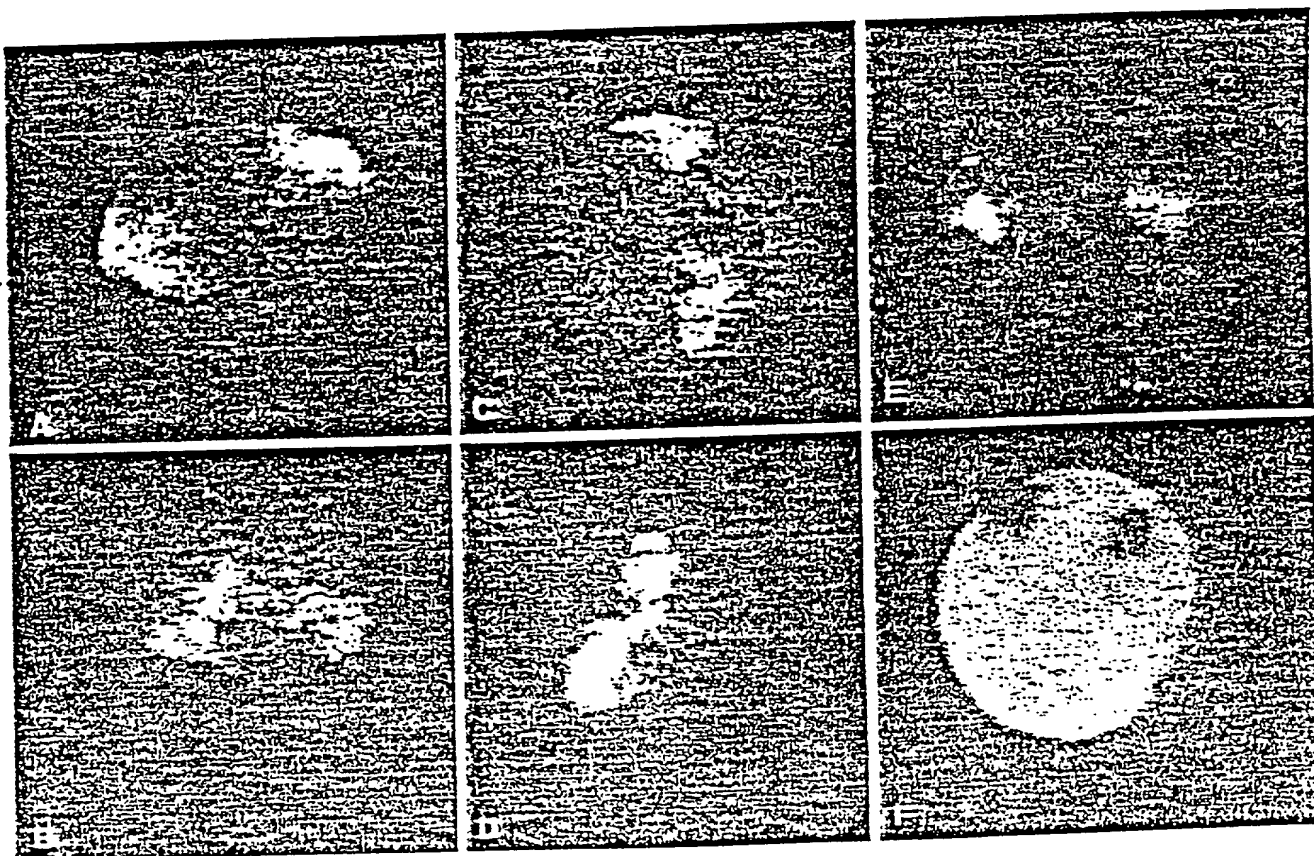




FIGURE 6

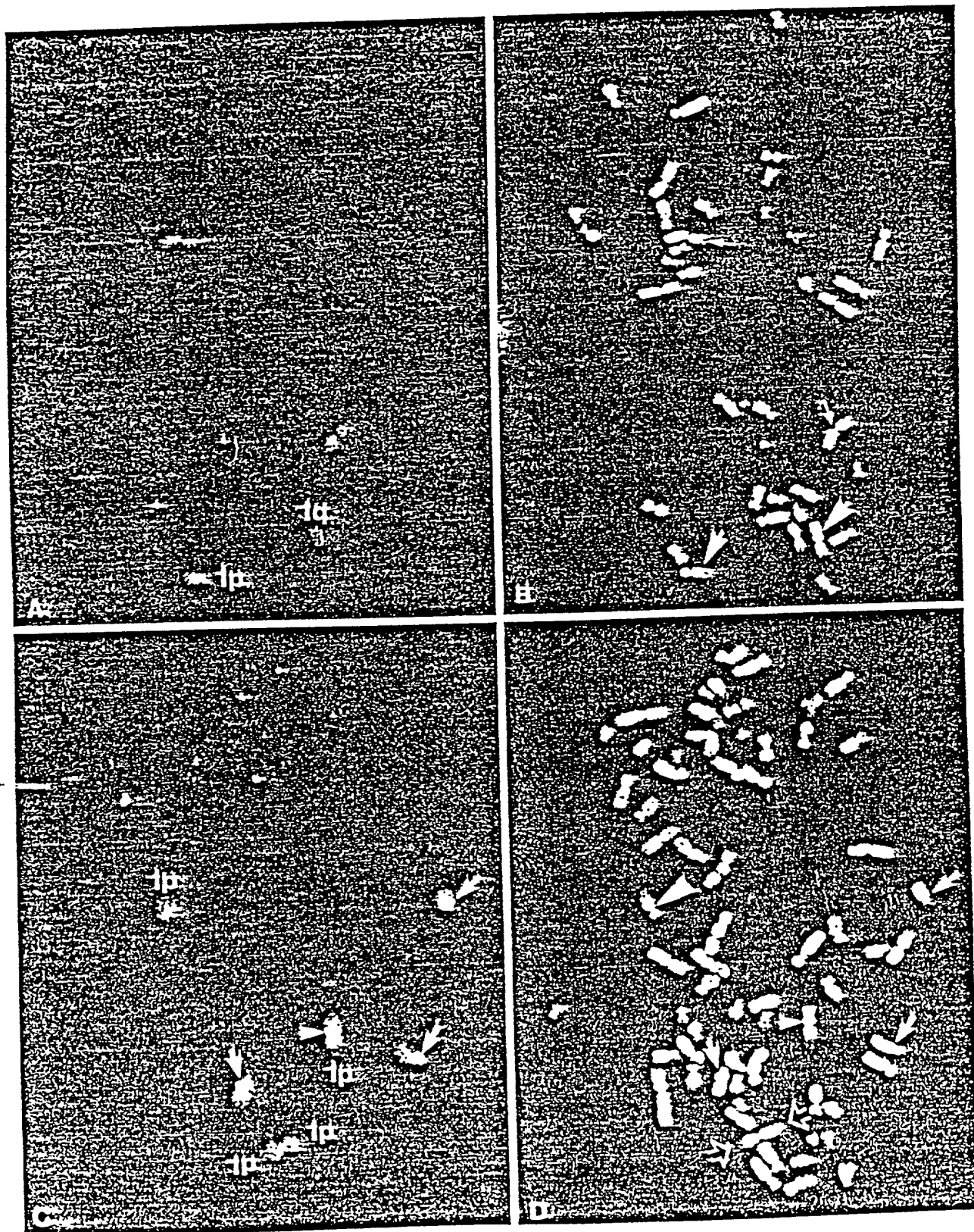


FIGURE 7

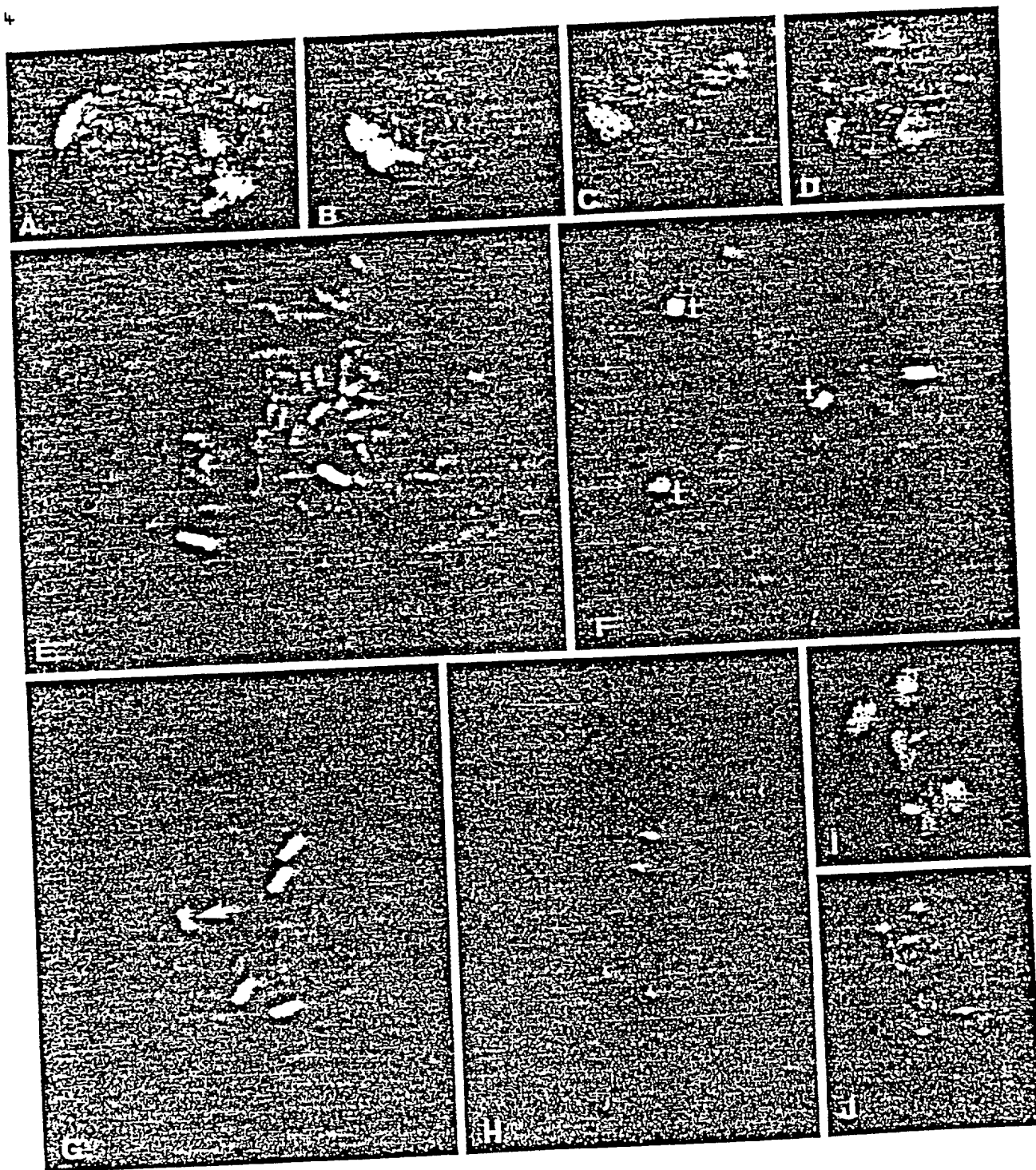




FIGURE 8

⑤

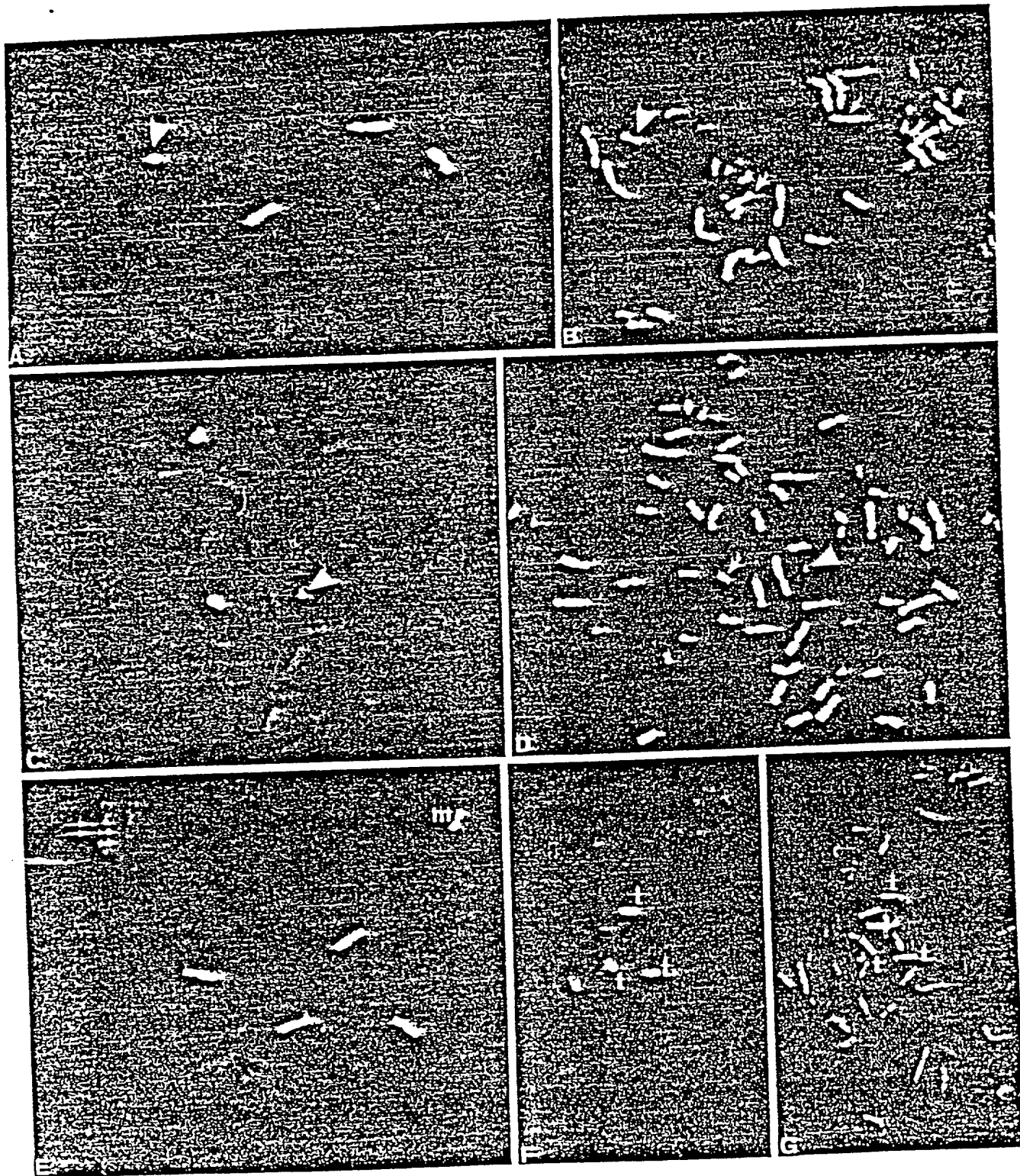


FIGURE 9

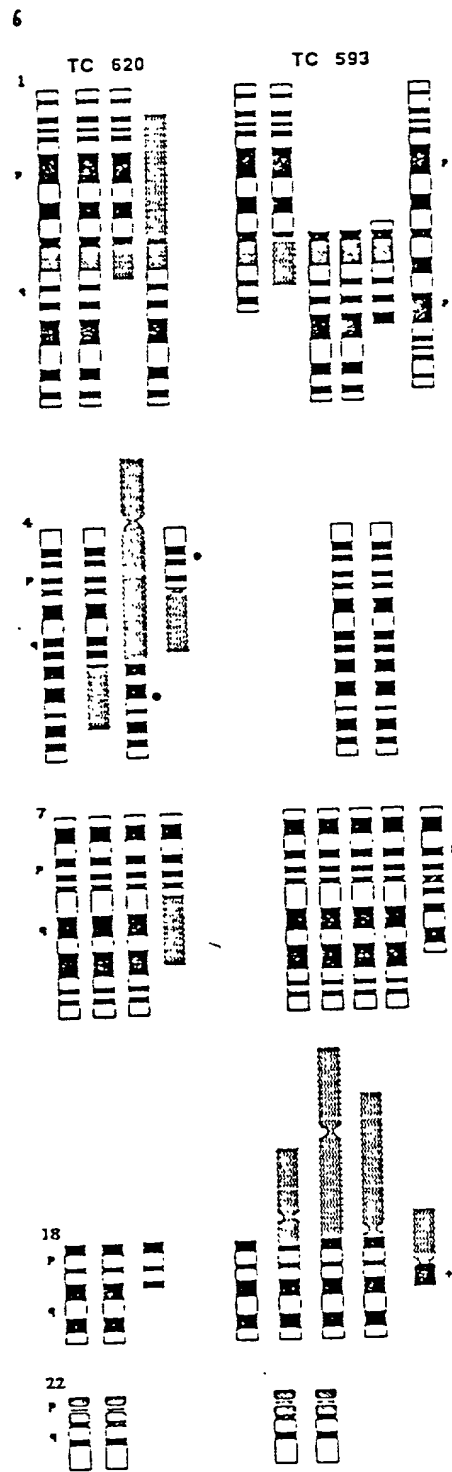
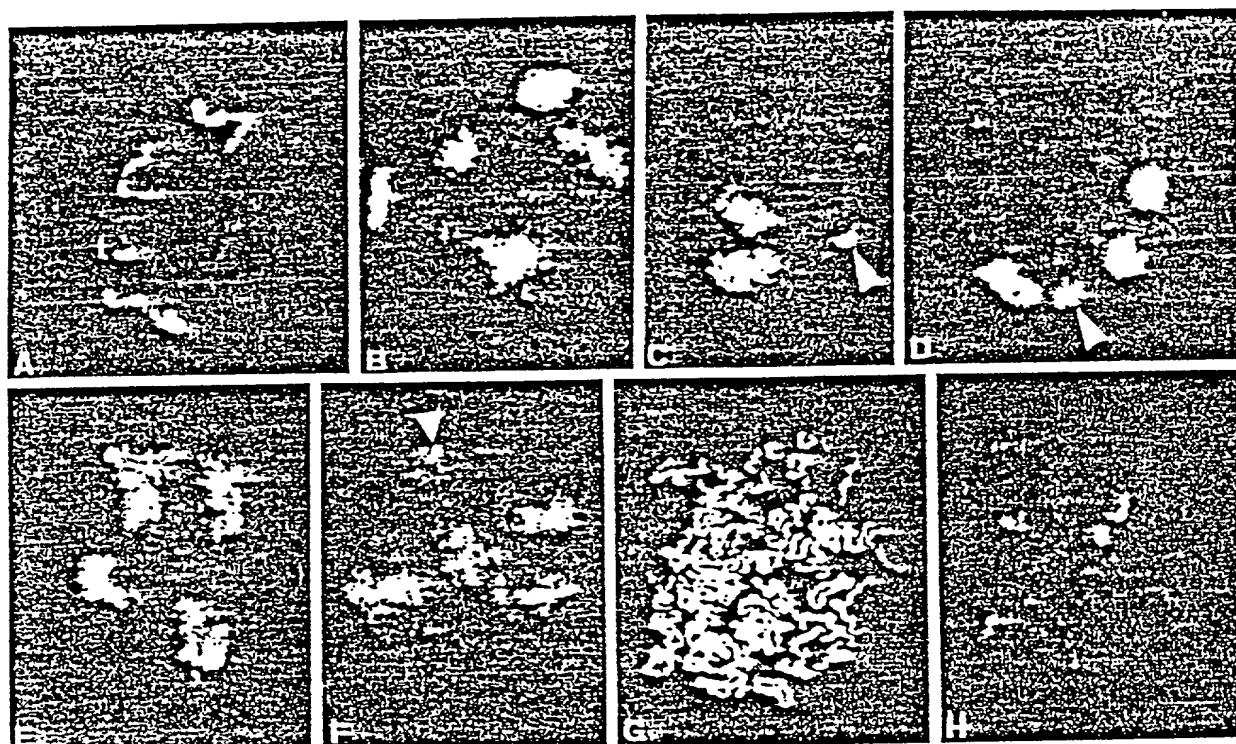


FIGURE 10



5-54 W.D.

FIGURE 11

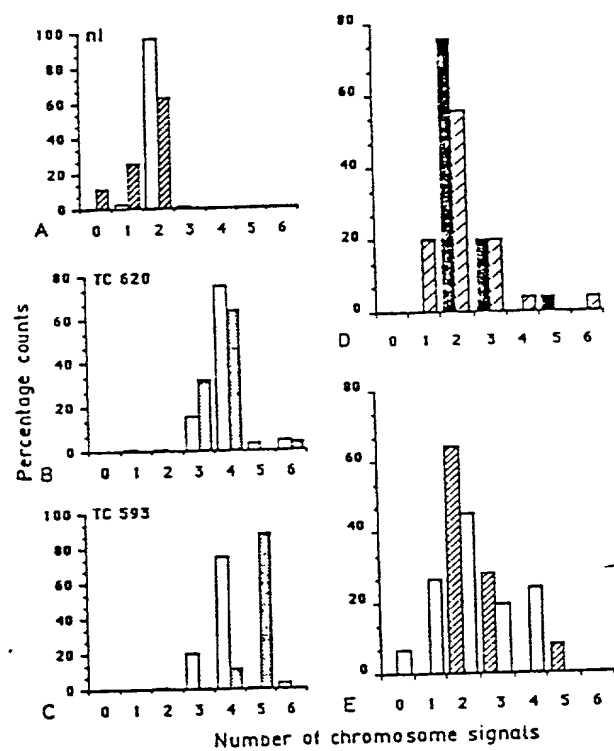


FIGURE 12

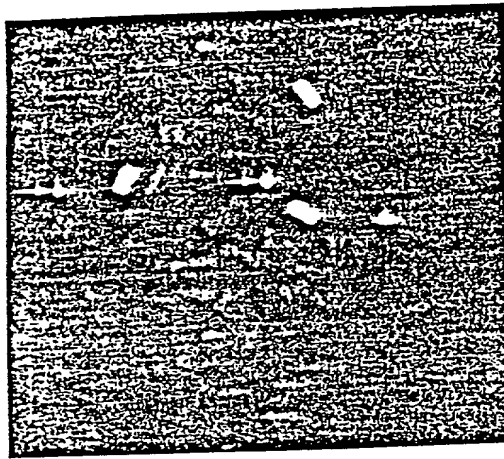


FIGURE 13

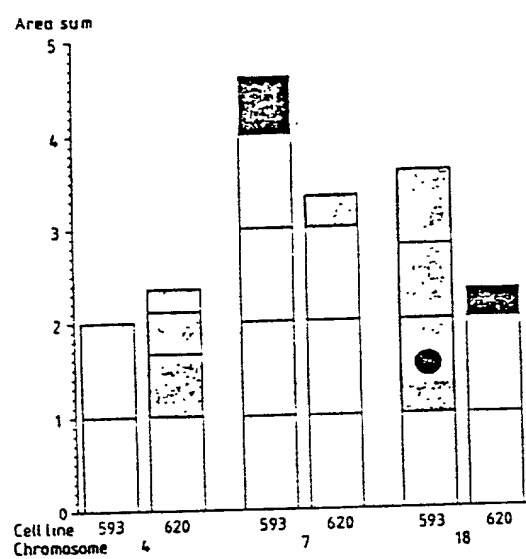
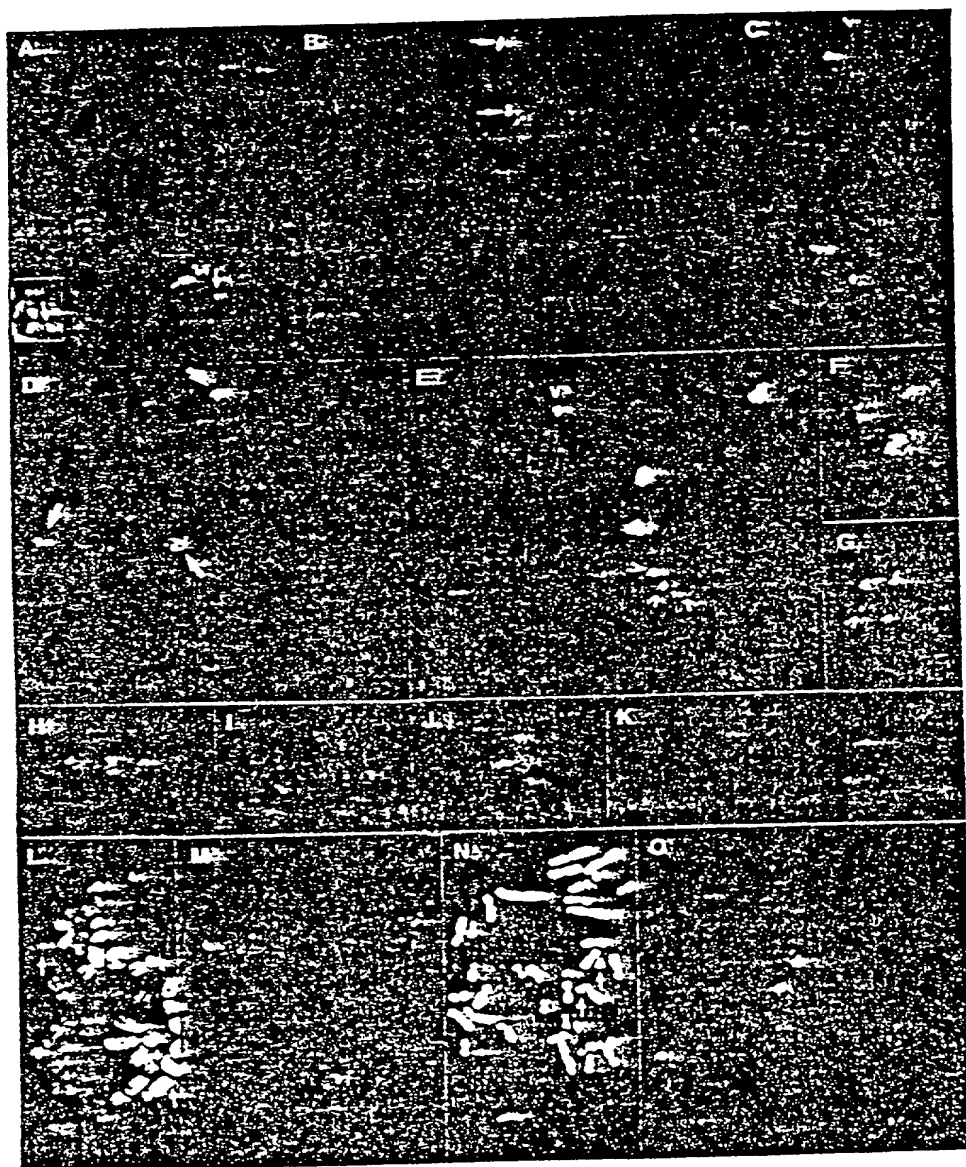


FIGURE 14



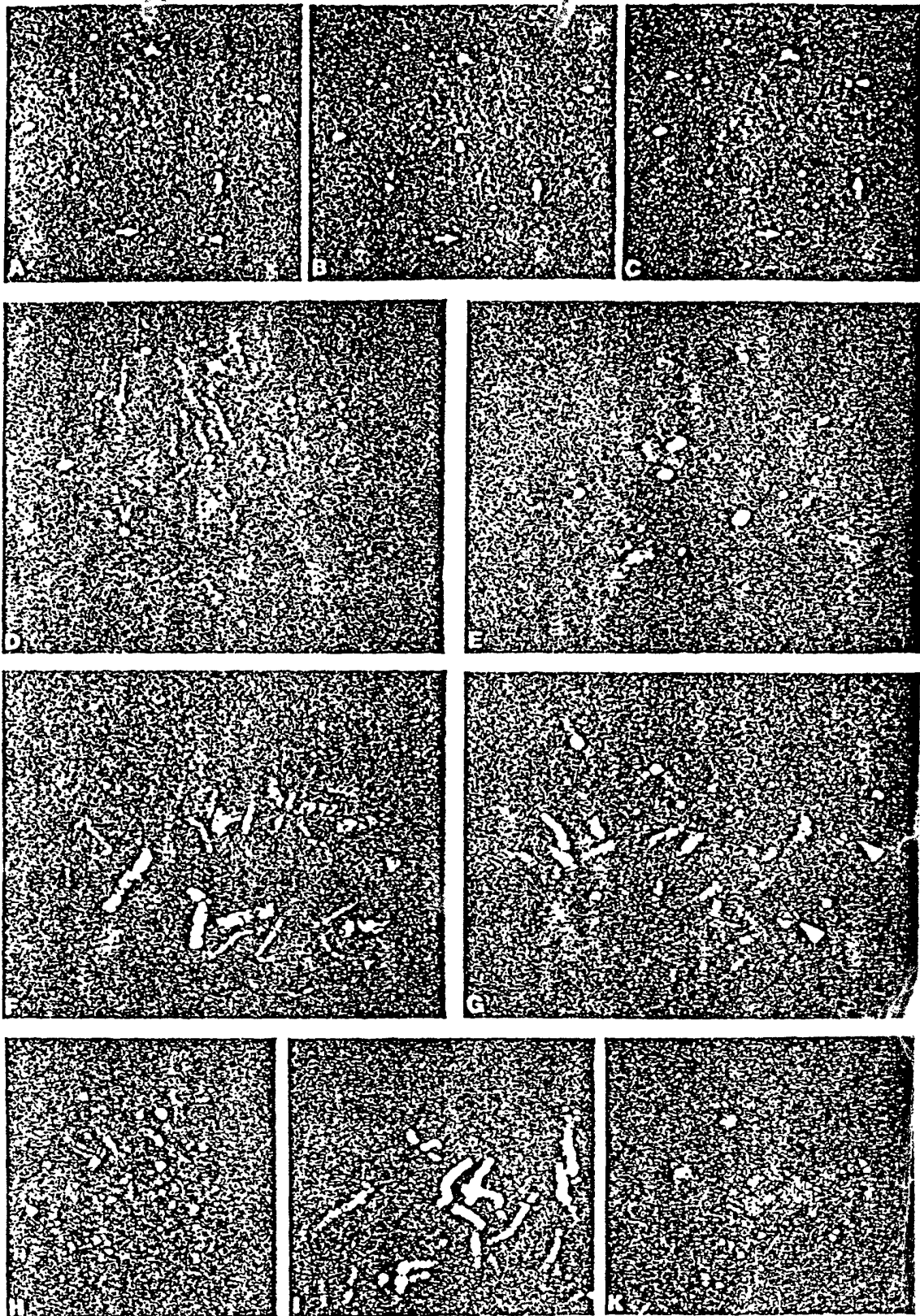


FIGURE 15



Declaration, Petition and Power of Attorney for  
File-Wrapper-Continuation Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated  
below next to my name,

I believe I am the original, first and sole inventor (if only  
one name is listed below) or an original, first and joint  
inventor (if plural names are listed below) of the subject  
matter which is claimed and for which a patent is sought on the  
invention entitled

DELINEATION OF INDIVIDUAL HUMAN CHROMOSOMES IN METAPHASE  
AND INTERPHASE CELLS BY IN SITU SUPPRESSION HYBRIDIZATION

the specification of which

(check one)

\_\_\_ is attached hereto.

X was filed on February 14, 1992 as  
Application Serial No. 07/837,664  
and was amended  
on February 14, 1992.  
(if applicable)

I do not know and do not believe that the subject matter of  
this application was ever known or used in the United States  
before my invention thereof or patented or described in any  
printed publication in any country before my invention thereof  
or more than one year prior to the date of this application,  
and that said subject matter has not been patented or made the  
subject of an issued inventor's certificate in any country  
foreign to the United States on an application filed by me or  
my legal representatives or assigns more than twelve months  
prior to the date of this application; that I acknowledge my  
duty to disclose information of which I am aware which is  
material to the examination of this application, that no  
application for patent or inventor's certificate on the subject  
matter of this application has been filed by me or my  
representatives or assigns in any country foreign to the United  
States, except those identified below, and that I have reviewed  
and understand the contents of the specification, including the  
claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is  
material to the examination of this application in accordance  
with Title 37, Code of Federal Regulations, §1.56(a).

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Check one:

☒ no such applications have been filed.

☐ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS  
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 37 USC 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS  
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application. As to subject matter of this application which is common to my earlier United States application, if any, described below, I do not believe that the same was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to said earlier application, or in public use or on sale in the United States more than one year prior to said earlier application, that the said common subject matter has not been patented or made the subject of an inventor's certificate issued before the date of said earlier application in any country foreign to the United States on an application, filed by me or my legal representatives or assigns more than twelve months prior to said application and that no application for patent or inventor's certificate on said subject matter has been filed by me or my representatives or assigns in any country foreign to the United States except those identified herein.

<u>07/271,609</u>	<u>November 15, 1988</u>	<u>Abandoned</u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented,pending,aband.)
<u>                    </u>	<u>                    </u>	<u>                    </u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented,pending,aband.)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

John A. Lahive, Jr.	Reg. No. 19,788	Michael I. Falkoff	Reg. No. 30,833
W. Hugo Liepmann	Reg. No. 20,407	Jeremiah Lynch	Reg. No. 17,425
James E. Cockfield	Reg. No. 19,162	Ann-Louise Kerner	Reg. No. 33,523
Mark G. Lappin	Reg. No. 26,618	James E. Maslow	Reg. No. 29,953
Thomas V. Smurzynski	Reg. No. 24,798	Giulio A. DeConti, Jr.	Reg. No. 31,503
Ralph A. Loren	Reg. No. 29,325	Ann Lamport Hammitte	Reg. No. 34,858
Thomas J. Engellenner	Reg. No. 28,711	Douglas J. Kline	Reg. No. 35,574
David J. Powsner	Reg. No. 31,868	Elizabeth A. Hanley	Reg. No. 33,505
William C. Geary III	Reg. No. 31,359	Amy E. Mandragouras	Reg. No. P-36,207

Send Correspondence to: Giulio A. DeConti, Jr. - Reg. No. 31,503  
LAHIVE & COCKFIELD  
60 State Street  
Boston, MA 02109

Direct Telephone Calls to: Giulio A. DeConti, Jr.  
(617) 227-7400

Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

David C. Ward  
Full name of sole or first inventor

Inventor's signature	Date
<u>89 Quail Run</u>	
<u>Madison, Connecticut 06443</u>	
Residence	
<u>United States of America</u>	
Citizenship	
<u>Post Office Address (if different)</u>	

Peter Lichter  
Full name of second inventor, if any  
Peter Lichter 7/7/92  
Inventor's signature Date  
Hauheckenweg 21  
D6900, Heidelberg, Germany  
Residence  
Germany  
Citizenship  
Post Office Address (if different)

Thomas Cremer  
Full name of third inventor, if any  
H. Thomas Cremer 7.7.92  
Inventor's signature Date  
Friedrich Weinbrenner Strasse H. Thomas Cremer  
D6900, Heidelberg, Germany  
Residence  
Germany  
Citizenship  
Post Office Address (if different)

Laura Manuelidis  
Full name of fourth inventor, if any  
Inventor's signature Date  
585 Ellsworth Avenue  
New Haven, Connecticut 06511  
Residence  
United States of America  
Citizenship  
Post Office Address (if different)

Thomas Ried  
Full name of fifth inventor, if any  
[Signature] 7/7/82  
Inventor's signature Date  
In den Pfädelsäckern 48  
D6900, Heidelberg, Germany  
Residence  
Germany  
Citizenship  
Post Office Address (if different)

Antonio Baldini  
Full name of sixth inventor, if any  
Inventor's signature Date  
Residence  
Italy  
Citizenship  
Post Office Address (if different)

Full name of seventh inventor, if any  
Inventor's signature Date  
Residence  
Citizenship  
Post Office Address (if different)

Declaration, Petition and Power of Attorney for  
File-Wrapper-Continuation Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated  
below next to my name,

I believe I am the original, first and sole inventor (if only  
one name is listed below) or an original, first and joint  
inventor (if plural names are listed below) of the subject  
matter which is claimed and for which a patent is sought on the  
invention entitled

DELINEATION OF INDIVIDUAL HUMAN CHROMOSOMES IN METAPHASE  
AND INTERPHASE CELLS BY IN SITU SUPPRESSION HYBRIDIZATION

the specification of which

(check one)

\_\_\_\_\_ is attached hereto.

X was filed on February 14, 1992 as  
Application Serial No. 07/837,664  
and was amended  
on February 14, 1992 (if applicable).

I do not know and do not believe that the subject matter of  
this application was ever known or used in the United States  
before my invention thereof or patented or described in any  
printed publication in any country before my invention thereof  
or more than one year prior to the date of this application,  
and that said subject matter has not been patented or made the  
subject of an issued inventor's certificate in any country  
foreign to the United States on an application filed by me or  
my legal representatives or assigns more than twelve months  
prior to the date of this application; that I acknowledge my  
duty to disclose information of which I am aware which is  
material to the examination of this application, that no  
application for patent or inventor's certificate on the subject  
matter of this application has been filed by me or my  
representatives or assigns in any country foreign to the United  
States, except those identified below, and that I have reviewed  
and understand the contents of the specification, including the  
claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is  
material to the examination of this application in accordance  
with Title 37, Code of Federal Regulations, §1.56(a).

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Check one:

☒ no such applications have been filed.

☐ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS  
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 37 USC 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS  
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION




CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application. As to subject matter of this application which is common to my earlier United States application, if any, described below, I do not believe that the same was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to said earlier application, or in public use or on sale in the United States more than one year prior to said earlier application, that the said common subject matter has not been patented or made the subject of an inventor's certificate issued before the date of said earlier application in any country foreign to the United States on an application, filed by me or my legal representatives or assigns more than twelve months prior to said application and that no application for patent or inventor's certificate on said subject matter has been filed by me or my representatives or assigns in any country foreign to the United States except those identified herein.

<u>07/271,609</u>	<u>November 15, 1988</u>	<u>Abandoned</u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented,pending,aband.)
<u>                    </u>	<u>                    </u>	<u>                    </u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented,pending,aband.)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

John A. Lahive, Jr.	Reg. No. 19,788	Michael I. Falkoff	Reg. No. 30,833
W. Hugo Liepmann	Reg. No. 20,407	Jeremiah Lynch	Reg. No. 17,425
James E. Cockfield	Reg. No. 19,162	Ann-Louise Kerner	Reg. No. 33,523
Mark G. Lappin	Reg. No. 26,618	James E. Maslow	Reg. No. 29,953
Thomas V. Smurzynski	Reg. No. 24,798	Giulio A. DeConti, Jr.	Reg. No. 31,503
Ralph A. Loren	Reg. No. 29,325	Ann Lamport Hammitte	Reg. No. 34,858
Thomas J. Engellenner	Reg. No. 28,711	Douglas J. Kline	Reg. No. 35,574
David J. Powsner	Reg. No. 31,868	Elizabeth A. Hanley	Reg. No. 33,505
William C. Geary III	Reg. No. 31,359	Amy E. Mandragouras	Reg. No. P-36,207

Send Correspondence to: Giulio A. DeConti, Jr. - Reg. No. 31,503  
LAHIVE & COCKFIELD  
60 State Street  
Boston, MA 02109

Direct Telephone Calls to: Giulio A. DeConti, Jr.  
(617) 227-7400

Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

David C. Ward  
Full name of sole or first inventor

David C. Ward  
Inventor's signature

7/13/72  
Date

89 Quail Run

Madison, Connecticut 06443

Residence

United States of America

Citizenship

Post Office Address (if different)

Peter Lichter

Full name of second inventor, if any

Inventor's signature

Date

Hauheckenweg 21

D6900, Heidelberg, Germany

Residence

Germany

Citizenship

Post Office Address (if different)

Thomas Cremer

Full name of third inventor, if any

Inventor's signature

Date

Friedrich Weinbrenner Strasse

D6900, Heidelberg, Germany

Residence

Germany

Citizenship

Post Office Address (if different)

Laura Manuelidis

Full name of fourth inventor, if any

*Laura Manuelidis*

Inventor's signature

Date

585 Ellsworth Avenue

New Haven, Connecticut 06511

Residence

United States of America

Citizenship

Post Office Address (if different)

7/13/92

Thomas Ried  
Full name of fifth inventor, if any

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
In den Pfadelsackern 48  
D6900 Heidelberg, Germany  
Residence \_\_\_\_\_  
Germany  
Citizenship \_\_\_\_\_  
Post Office Address (if different) \_\_\_\_\_

Antonio Baldini  
Full name of sixth inventor, if any

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
Residence \_\_\_\_\_  
Italy  
Citizenship \_\_\_\_\_  
Post Office Address (if different) \_\_\_\_\_

Full name of seventh inventor, if any

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
Residence \_\_\_\_\_  
Citizenship \_\_\_\_\_  
Post Office Address (if different) \_\_\_\_\_

Attorney's  
Docket Number: IGI-001CN

Declaration, Petition and Power of Attorney for  
File-Wrapper-Continuation Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated  
below next to my name,

I believe I am the original, first and sole inventor (if only  
one name is listed below) or an original, first and joint  
inventor (if plural names are listed below) of the subject  
matter which is claimed and for which a patent is sought on the  
invention entitled

DELINEATION OF INDIVIDUAL HUMAN CHROMOSOMES IN METAPHASE  
AND INTERPHASE CELLS BY IN SITU SUPPRESSION HYBRIDIZATION

the specification of which

(check one)

\_\_\_ is attached hereto.

X was filed on February 14, 1992 as  
Application Serial No. 07/837,664  
and was amended  
on February 14, 1992.  
(if applicable)

I do not know and do not believe that the subject matter of  
this application was ever known or used in the United States  
before my invention thereof or patented or described in any  
printed publication in any country before my invention thereof  
or more than one year prior to the date of this application,  
and that said subject matter has not been patented or made the  
subject of an issued inventor's certificate in any country  
foreign to the United States on an application filed by me or  
my legal representatives or assigns more than twelve months  
prior to the date of this application; that I acknowledge my  
duty to disclose information of which I am aware which is  
material to the examination of this application, that no  
application for patent or inventor's certificate on the subject  
matter of this application has been filed by me or my  
representatives or assigns in any country foreign to the United  
States, except those identified below, and that I have reviewed  
and understand the contents of the specification, including the  
claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is  
material to the examination of this application in accordance  
with Title 37, Code of Federal Regulations, §1.56(a).

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Check one:

- ☒ no such applications have been filed.  
☐ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS  
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 37 USC 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS  
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

---

---

---

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application. As to subject matter of this application which is common to my earlier United States application, if any, described below, I do not believe that the same was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to said earlier application, or in public use or on sale in the United States more than one year prior to said earlier application, that the said common subject matter has not been patented or made the subject of an inventor's certificate issued before the date of said earlier application in any country foreign to the United States on an application, filed by me or my legal representatives or assigns more than twelve months prior to said application and that no application for patent or inventor's certificate on said subject matter has been filed by me or my representatives or assigns in any country foreign to the United States except those identified herein.

<u>07/271,609</u> (Application Serial No.)	<u>November 15, 1988</u> (Filing Date)	<u>Abandoned</u> (Status) (patented, pending, aband.)
<u>                    </u> (Application Serial No.)	<u>                    </u> (Filing Date)	<u>                    </u> (Status) (patented, pending, aband.)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

John A. Lahive, Jr.	Reg. No. 19,788	Michael I. Falkoff	Reg. No. 30,833
W. Hugo Liepmann	Reg. No. 20,407	Jeremiah Lynch	Reg. No. 17,425
James E. Cockfield	Reg. No. 19,162	Ann-Louise Kerner	Reg. No. 33,523
Mark G. Lappin	Reg. No. 26,618	James E. Maslow	Reg. No. 29,953
Thomas V. Smurzynski	Reg. No. 24,798	Giulio A. DeConti, Jr.	Reg. No. 31,503
Ralph A. Loren	Reg. No. 29,325	Ann Lamport Hammitte	Reg. No. 34,858
Thomas J. Engellenner	Reg. No. 28,711	Douglas J. Kline	Reg. No. 35,574
David J. Powsner	Reg. No. 31,868	Elizabeth A. Hanley	Reg. No. 33,505
William C. Geary III	Reg. No. 31,359	Amy E. Mandragouras	Reg. No. P-36,207

Send Correspondence to: Giulio A. DeConti, Jr. - Reg. No. 31,503  
LAHIVE & COCKFIELD  
60 State Street  
Boston, MA 02109

Direct Telephone Calls to: Giulio A. DeConti, Jr.  
(617) 227-7400

Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

David C. Ward  
Full name of sole or first inventor

Inventor's signature	Date
<u>89 Quail Run</u>	
<u>Madison, Connecticut 06443</u>	
Residence	
<u>United States of America</u>	
Citizenship	
Post Office Address (if different)	



Peter Lichter

Full name of second inventor, if any

Inventor's signature

Date

Hauheckenweg 21

D6900, Heidelberg, Germany

Residence

Germany

Citizenship

Post Office Address (if different)

Thomas Cremer

Full name of third inventor, if any

Inventor's signature

Date

Friedrich Weinbrenner Strasse

D6900, Heidelberg, Germany

Residence

Germany

Citizenship

Post Office Address (if different)

Laura Manuelidis

Full name of fourth inventor, if any

Inventor's signature

Date

585 Ellsworth Avenue

New Haven, Connecticut 06511

Residence

United States of America

Citizenship

Post Office Address (if different)

Thomas Ried  
Full name of fifth inventor, if any

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
In den Pfadelsackern 48  
D6900 Heidelberg, Germany  
Residence \_\_\_\_\_  
Germany  
Citizenship \_\_\_\_\_  
Post Office Address (if different) \_\_\_\_\_

Antonio Baldini  
Full name of sixth inventor, if any  
Alberto Baldini July 8, 1992  
Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
5 St. Michaels Garden  
London W10 England  
Residence \_\_\_\_\_  
Italy  
Citizenship \_\_\_\_\_  
Post Office Address (if different) \_\_\_\_\_

Full name of seventh inventor, if any

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
Residence \_\_\_\_\_  
Citizenship \_\_\_\_\_  
Post Office Address (if different) \_\_\_\_\_

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of: D. Ward *et al.*

Serial No.: 08/312,429

Filed: September 26, 1994

For: DELINEATION OF INDIVIDUAL HUMAN  
CHROMOSOMES IN METAPHASE AND INTERPHASE  
CELLS BY *IN SITU* SUPPRESSION HYBRIDIZATION

Attorney Docket No: IGI-001CN2

Group Art Unit: 1805

Examiner: S. Priebe

COPY

Assistant Commissioner for Patents  
Washington, D.C. 20231

Certificate of Transmission by Facsimile

I hereby certify that this correspondence is being transmitted by facsimile to: Assistant Commissioner for Patents, Washington, D.C. 20231 at (703) 308-6916 on the date set forth below.

23 November 1998  
Date of Signature and of Mail Deposit

By:

Jeanne M. DiGiorgio  
Jeanne M. DiGiorgio  
Reg. No. 41,710  
Attorney for Applicants

ASSOCIATE POWER OF ATTORNEY

Dear Sir:

The undersigned attorney has the power of attorney in the subject application. He hereby grants an associate power to:

Jeanne M. DiGiorgio  
Registration No. 41,710  
Lahive & Cockfield, LLP  
28 State Street  
Boston, MA 02109

Please continue to forward all written and telephonic communications to Giulio A. DeConti, Jr., Esq. at the address and telephone number listed below.

Respectfully submitted,

Thomas V. Smurzynski  
Thomas V. Smurzynski  
Registration No. 24,798  
Attorney for Applicants

LAHIVE & COCKFIELD, LLP  
28 State Street  
Boston, MA 02109  
Tel. (617) 227-7400  
Dated: November 23, 1998